NEWBORN SCREENING FOR PRIMARY IMMUNODEFICIENCY DISEASES

Thesis submitted for the degree of Doctor of Philosophy

Dr Jovanka Rosslyn King

FRACP FRCPA BMBS (Hons.) B. Pod DCH (SA)

Department of Paediatrics & Child Health
School of Medicine & the Robinson Research Institute
University of Adelaide
Adelaide, South Australia

Department of Immunopathology
SA Pathology
The Women's & Children's Hospital
North Adelaide,
South Australia

Division of Clinical Immunology
Department of Laboratory Medicine
Karolinska University Hospital
Karolinska Institutet
Stockholm, Sweden

September 2018

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER 8

LIST OF FIGURES

CHAPTER 8

ABSTRACT

Primary immunodeficiency diseases (PID) are a heterogeneous group of inborn errors of immunity, resulting in significant morbidity and mortality in affected infants and young children. To date, over 320 distinct genetic abnormalities resulting in PID have been described. Of interest to this thesis is severe combined immunodeficiency (SCID), where survival and long-term outcomes are significantly improved if haematopoietic stem cell transplantation (HSCT) is performed prior to 3.5 months of age. Hence, early detection of affected individuals with PID is of critical importance. The ideal infant screening strategy for PID remains controversial.

Most established screening programs utilise a T cell receptor excision circle (TREC) assay to screen newborns for SCID. TREC are surrogate markers for thymic T cell output, with low levels indicative of T cell lymphopaenia. B cell lymphopaenia can also be detected through quantification of kappa-deleting recombination excision circles (KREC). This thesis presents the results of a two-year, prospective screening study for severe forms of PID manifested by T and/or B cell lymphopaenia. This was the first large-scale screening study using a multiplexed TREC/KREC assay to simultaneously detect T and B cell lymphopaenia. This assay was used to screen 58,834 newborns, resulting in the identification of three infants with PID. The findings of this study confirm the efficacy of DNA-based screening strategies to identify infants with PID and highlight the additional benefits of simultaneous screening for T and B cell lymphopaenia.

TREC and KREC assays have predominantly been used in the context of newborn screening for PID, with limited application of these assays beyond the newborn period. This thesis investigated the utility of these assays in the clinical evaluation of older patients with suspected PID. The results showed that KREC levels remain low or undetectable throughout life in patients with X-linked agammaglobulinaemia (XLA). These findings, therefore, demonstrate a wider application and diagnostic utility of these assays in older children and adults.

Hypogammaglobulinaemia is a feature of several forms of PID, including primary antibody deficiency disorders and combined immunodeficiencies such as SCID. Currently, there is no strategy by which to screen infants for hypogammaglobulinaemia, and measuring antibody levels in infants is confounded by the presence of maternal antibodies. This thesis presents the results of a small pilot study evaluating a transcriptomic approach to the identification of children with hypogammaglobulinaemia. Our findings show that RNA can be successfully extracted from dried bloods spots, and that immunoglobulin heavy chain gene expression assays are useful to analyse the immunoglobulin transcriptome of healthy individuals and

patients with PID. In addition, this assay is able to identify children with complete deficiencies of immunoglobulin production (XLA and X-linked hyper-IgM syndrome).

In approaching the era of personalised medicine, it would be advantageous not only to screen individuals for disease states, but also to identify augmentable disease susceptibility factors, thereby enabling prevention of disease. Knowledge of susceptibility factors enables the provision of patient-specific advice regarding the need for screening, therapy or prophylaxis. This thesis describes a newborn screening strategy which identifies specific SNPs in fucosyltransferase (*FUT2* and *FUT3*) genes which determine the expression of histo-blood group antigens (HBGA) (H type 1 and Lewis). These HBGA are important for microbial attachment and metabolism and, therefore, influence an individual's predisposition to selected infectious diseases (including rotavirus and norovirus). They also play a role in shaping the microbiome and have been associated with the development of autoimmune and inflammatory diseases. In this thesis, a genotyping method was developed to determine the frequency of SNPs in *FUT2* and *FUT3*, and the ensuing Lewis b and secretor status. Our results demonstrated that a higher than expected proportion of newborns in the cohort were non-secretors and Lewis b negative, predisposing them to specific infections and other diseases, with potential implications for vaccination.

This thesis contributes to the literature regarding newborn screening strategies for PID, providing evidence for the efficacy of combined TREC/KREC screening to identify infants with severe forms of PID manifested by T and/or B cell lymphopaenia. It outlines the utility of screening tests beyond the newborn period as tools for the diagnostic work-up of patients with PID. Evidence is provided for the utility of a newborn screening approach for SNPs conferring disease susceptibility, which has implications for the provision of personalised, precision medicine. These findings are discussed and reviewed with respect to the current international status of newborn screening for PID, highlighting differences in screening approaches. This thesis reviews application of other methods, including protein-based assays and targeted sequencing to expand the range of currently screened diseases. The potential utility of screening newborns using up-front next generation sequencing (whole exome and whole genome sequencing) is discussed, highlighting the key considerations prior to adopting this approach.

DECLARATION

I certify that this work is original and has not been accepted for the award of any other degree or diploma in any university or other tertiary institution and, contains no material previously published or written by another person, except where due acknowledgement is made in the text.

I certify that no part of the work will be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of the degree except where due reference has been made in the text.

I give consent for the thesis to be made available for loan and photocopying after it has been examined and placed in the library, subject to the provisions of the Copyright Act 1968.

I give consent for the digital version of the thesis to be made available on the web, via the University's digital research repository, the library catalogue and also through web search engines.

I acknowledge that copyright of published works contained within this thesis resides with the copyright holder(s) of those works.

I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship. I also acknowledge the support of the Royal College of Pathologists of Australasia (RCPA) Foundation (2016 Mike and Carole Ralston Travelling Fellowship).

Dr Jovanka King

6 September 2018

ACKNOWLEDGEMENTS

I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship and the Royal College of Pathologists of Australasia (RCPA) Foundation (2016 Mike and Carole Ralston Travelling Fellowship).

I would like to acknowledge the guidance and assistance of my local supervisors, Professors Antonio Ferrante and Michael Gold. Tony, I thank you for all the opportunities you have given me, and for supporting my local projects and laboratory work. I am grateful to you for encouraging me to embark upon this path, and I appreciate the support you have shown me along the way. Mike, I would like to thank you not only for your guidance throughout my candidature, but also for the mentorship you have provided me over the past several years in both clinical and academic spheres. I am grateful for your insights and ongoing support.

I wish to thank my supervisor from the Karolinska Institutet, Professor Lennart Hammarström, for his vision, mentorship and guidance in undertaking these projects. Lennart, stort tack för ditt stöd och mentorskap de senaste tre åren och för varje tillfälle du har gett mig att utveckla mig själv och min vetenskapliga utbildning. Med din vägledning har jag uppnått så många saker som annars inte skulle ha varit möjliga, och för det är jag verkligen tacksam. Din expertis och ditt engagemang för ditt arbete är inspirerande, och det har varit ett nöje att jobba med dig. Du har lärt mig att allt är möjligt, och att alla problem kan lösas. Jag ser fram emot att fortsätta vårt gemensamma arbete i framtiden. Du har betytt mer för mig än vad du antagligen förstår, för det, tack!

I wish to thank my collaborators and co-authors, especially Stephan Borte, for willingly sharing their knowledge and expertise - it has been an honour to work with such esteemed colleagues. In particular, I would like to thank my friend and colleague, Jezabel Varadé-Lopez, who I am indebted to for all her assistance, and for so willingly sharing her expertise. Jezabel, palabras no pueden expresar lo agradecida que estoy por todo lo que has hecho por mí – tú me has enseñado muchas habilidades prácticas que "científica verdadera"! Yo he sido inspirada por tu sabiduría y habilidades, y por lo generosa que has sido con tu tiempo para enseñar y ayudar a otros. Este trabajo no hubiera sido posible sin tu ayuda, y por esto te estaré eternamente agradecida. Pero más que todo, te agradezco por tu amistad, y espero podamos compartir más aventuras juntas en el futuro!

I wish to thank past and present members of the Hammarström Laboratory group at the Karolinska Institutet, Pan, Kerstin, Rozina, CK, Harold, Yin, Konstantinos, Andrea, Omar, Hassan, Xiao Gan, Radhika, Xiao Fei, Fang Lei, Marco, Bo, Hui, Zhu, Wei, Sofia, Rosa,

Kasper, Moa, Lisa, Luke, Chloe, Monika and Annica. Thank you for making my time in Sweden so memorable and full of so many fantastic events, trips and celebrations - I am so privileged to have such a wonderful, big international family: tack så mycket – muchas gracias – tak - grazie mille - 谢谢 – ευχαριστώ – merci beaucoup - आपका बहुत बहुत धन्यवाद - متشكر – . شكر

I would like to acknowledge my colleagues and friends in the Ferrante Laboratory in the Department of Paediatric Immunopathology at the Women's & Children's Hospital. In particular, I would like to thank Alexander Quach and David Shields for their technical assistance in the laboratory and scientific discussions. I would also like to acknowledge the support and assistance of Dr Patrick Quinn, Head of Department and my medical, nursing and administration colleagues in the Department of Allergy and Clinical Immunology at the Women's & Children's Hospital.

My patients and their families are, and will always, remain central to all my clinical, laboratory and research efforts. They provide me with both the inspiration and motivation to continue to work towards achieving improved health outcomes through research and development activities. I would like to acknowledge the participation of our patients and their families in these projects. I sincerely thank them for their enthusiasm and willingness to participate in order to help other families facing similar challenges to travel an easier path in the future.

I would like to acknowledge the endless support of my strong network of family, friends and colleagues, near and far. A special thank you to Rikki and Asha for always being there for me, for all their encouragement and for over a decade of 'Tier 1' friendship and adventure.

I would like to acknowledge the unfaltering love and support of my family, without whom these achievements would not have been possible. To my mother, Branka and sister, Svetlana – you are the strongest and wisest women I know. Mum, there will never be enough words to explain how much you mean to me. You are the greatest role model, and we have become the people we are because of the life that you and Dad created for us. You have always put us first and encouraged us to follow our dreams. You are always there to celebrate our triumphs and pick us up when we fall. Svetlana, I am so lucky to have you not only as my sister, but as my best friend. Thank you for your assistance with editing and formatting my thesis, and for all your support in both academia and in life – I don't know what I would do without you! You always strive for excellence and have achieved so much, and are a true inspiration to everyone around you. Thank you for always being by my side.

Ross William King (1945 – 2014)

My father, Ross, was good at everything he turned his hand to, and inspired me to challenge myself, and take every opportunity to embark on a new experience or learn a new skill. He taught me that a task started should be seen through to completion, and that the greatest rewards come from hard work and perseverance. I miss him, and all the good he brought to the world every day. I dedicate this thesis to his memory.

PUBLICATIONS

I. **King J** & Hammarström L, 'Newborn Screening for Primary Immunodeficiency Diseases**:** History, current and future practice, Journal of Clinical Immunology, epub Nov 8, doi: 10.1007/s10875-017-0455-x.

II. Barbaro M, Ohlsson A, Borte S, Jonsson S, Zetterström R, **King J**, Winiarski J, von Dobeln U, Hammarström L, 'Newborn screening for severe primary immunodeficiency diseases in Sweden– a two-year pilot TREC & KREC screening study', Journal of Clinical Immunology, 37(1), 51–60, doi.org/10.1007/s10875-016-0347-5.

III. **King J**, Borte S, Brodszki N, Von Dobeln U, Smith E, Hammarström L, 'Kappa recombining excision circle levels remain low or absent throughout life in patients with X-linked agammaglobulinemia', Paediatric Allergy & Immunology, doi.org/10.1111/pai.12893.

IV. **King J**, Varadé J, Hammarström L, **'**Fucosyltransferase gene polymorphisms and Lewis b negative status are frequent in Swedish newborns, with implications for infectious disease susceptibility and personalized medicine', Journal of the Pediatric Infectious Diseases Society, 9 December 2018, doi-org.proxy.kib.ki.se/10.1093/jpids/piy085

V. **King J**, Ludvigsson J, Hammarström L, 'Newborn Screening for Primary Immunodeficiency Diseases: The past, the present and the future', International Journal of Newborn Screening, International Journal of Neonatal Screening 2017, 3(3), 19, doi:10.3390/ijns3030019.

UNPUBLISHED MANUSCRIPT

King J, 'A transcriptomic approach to newborn screening for hypogammaglobulinaemia', unpublished manuscript.

PRESENTATIONS

A list of presentations delivered during candidature are listed below. Abstracts and other details are included in Appendix I.

I. **'Newborn screening for primary immunodeficiency: The next generation'**
Abstract published in Pathology (2018) 50(S1), p. S29.
https://doi.org/10.1016/j.pathol.2017.12.066
Invited speaker, Genetics Stream
Royal College of Pathologists of Australasia (RCPA) Pathology Update Meeting, Sydney, New South Wales, March 2018

II. **'Newborn screening for primary immunodeficiency: TREC, KREC and beyond'**
Abstract published in Pathology (2018) 50(S1), p. S45.
https://doi.org/10.1016/j.pathol.2017.12.108
Invited speaker, Immunology Stream
Royal College of Pathologists of Australasia (RCPA) Pathology Update Meeting, Sydney, New South Wales, March 2018

III. **'Case studies in paediatric primary immunodeficiency'**
Invited speaker
Australasian Society for Clinical Immunology and Allergy (ASCIA) SA Branch Meeting, February 2018

IV. **'Newborn screening for primary immunodeficiency diseases'**
Invited speaker
SA Pathology Colloquium, Adelaide
November 2017

V. **'Newborn screening for primary immunodeficiency'**
Invited speaker
Australasian Society for Clinical Immunology and Allergy (ASCIA) SA Branch Meeting, April 2017

RESEARCH IMPACT

The research included in this thesis was publicised in the public domain. A media release was generated by the Public Relations team at the Royal College of Pathologists of Australasia (RCPA) in the context of the 2018 Pathology Update meeting where these findings were presented. A summary of the uptake and dissemination of research findings by various print, online and radio media outlets is provided in Appendix II.

GRANTS AND AWARDS

I. **Australian Government Research Training Program Scholarship**
Australian Government
PhD candidature scholarship
Awarded September 2015
Value: $AUD 26,000 per annum for 3 years

II. **Mike and Carole Ralston Travelling Fellowship 2016**
Royal College of Pathologists of Australasia (RCPA) Foundation
Support of collaborative research activities in Sweden
Awarded February 2016
Value: $AUD 30,000

III. **School of Medicine Travel Grant 2016**
School of Medicine, University of Adelaide
Support for attendance of International Congress of Immunology, Melbourne, Victoria,
Awarded August 2016
Value: $AUD 2000

IV. **Special Purposes Fund Conference Support**
SA Pathology
Support for attendance of RCPA Pathology Update Meeting, Sydney, New South Wales
Awarded March 2018
Value: $AUD 1600

LIST OF ABBREVIATIONS

ACTB	Beta-actin (gene product)
ACTB	Beta-actin (gene)
ADA	Adenosine deaminase (protein)
ADA	Adenosine deaminase (gene)
AT	Ataxia telangiectasia
ATM	Ataxia telangiectasia mutated (gene)
BTK	Bruton's tyrosine kinase (protein)
BTK	Bruton's tyrosine kinase (gene)
CVID	Common variable immune deficiency
DBS	Dried blood spot
DNA	Deoxyribonucleic acid
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IGHA1	Immunoglobulin A heavy chain 1 (gene)
IGHG1	Immunoglobulin G heavy chain 1 (gene)
IGHM	Immunoglobulin M heavy chain (gene)
IgM	Immunoglobulin M
FACS	Fluorescence-activated cell sorting
FHL	Familial haemophagocytic lymphohistiocytosis
FUT	Fucosyltransferase
FUT2	Fucosyltransferase 2 (gene)
FUT3	Fucosyltransferase 3 (gene)

HBGA	Human blood group antigen
HSCT	Haematopoeitic stem cell transplantation
MHC	Major histocompatibility complex
NBS	Nijmegen breakage syndrome
NGS	Next-generation sequencing
KREC	Kappa-deleting recombination excision circle
PCR	Polymerase chain reaction
PID	Primary immunodeficiency disease
PKU	Phenylketonuria
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative real-time-polymerase chain reaction
RNA	Ribonucleic acid
SAD	Specific antibody deficiency
SCID	Severe combined immunodeficiency
SNP	Single nucleotide polymorphism
THI	Transient hypogammaglobulinaemia of infancy
TCR	T cell receptor
TREC	T cell receptor excision circle
V(D)J	Variable (diversity) junctional (recombination)
WAS	Wiskott-Aldrich syndrome
WES	Whole exome sequencing
WGS	Whole genome sequencing
X-HIGM	X-linked hyper IgM syndrome

XLA	X-linked agammaglobulinaemia
ZAP70	Zeta-chain-associated protein kinase 70 (protein)
ZAP70	Zeta-chain-associated protein kinase 70 (gene)

CHAPTER 1: INTRODUCTION

1.1 Contextual Statement and Targeted Literature Review

The case for newborn screening for primary immunodeficiency diseases

Primary immunodeficiency diseases (PID) are a heterogeneous group of inborn errors of immunity which typically present in infancy and childhood. To date, over 320 different molecular causes of PID have been identified[1]. Affected children suffer from recurrent, severe or unusual infections, resulting in increased morbidity, health system utilisation and in some cases, death. Diagnosing children with PID is frequently delayed, due to a lack of awareness of these conditions by health professionals, and lengthy clinical and laboratory diagnostic processes. Delayed diagnosis portends a poorer prognosis, given that by the time a diagnosis is eventually made, children are significantly unwell due to severe complications of untreated disease. Hence, there is a clear case for newborn screening for severe forms of PID, where early diagnosis and treatment can be life-saving and significantly reduce long-term morbidity[2-6].

Severe combined immunodeficiency (SCID) is a particularly catastrophic form of PID which is manifested by absent or reduced T cells, with variable presence of B and NK cells depending on the underlying molecular genetic defect[1]. Without definitive treatment with haematopoietic stem cell transplantation (HSCT), SCID is uniformly fatal. Evidence suggests that survival and long-term outcomes are significantly improved if HSCT is performed prior to 3.5 months of age[6]. Hence, early detection of infants with PID is critically important. The only realistic way to achieve the goal of early HSCT for SCID is by identifying affected infants soon after birth, in population-based newborn screening programs.

PID were previously thought to be rare entities; however results of international newborn screening studies provide evidence that disease frequency is much higher than initially thought. The incidence of SCID has been determined to be 1/58,000, and 1/7,300 infants will have T cell lymphopaenia due to other causes[7]. It is, therefore, highly likely that many cases of SCID have been missed in the past, with affected infants dying without their disease being recognised. This further strengthens the case for systematic newborn screening for these conditions.

Current scope of identifiable diseases and methods for newborn screening for severe forms of primary immunodeficiency

Newborn screening programs aim to identify infants with a range of severe conditions which are asymptomatic at birth, for which curative treatment is available[8]. The ideal strategy by which to screen infants for PID remains controversial, with various approaches having been adopted in different centres around the world. Current screening practices utilise a DNA-based method which measures surrogate markers of T cell production (T cell receptor excision circles, TREC)[9] in order to identify infants with T cell lymphopaenia. At the present time, most screening centres worldwide have adopted TREC-only assays to identify SCID as the target screened condition. More recently, a method has been established to enable simultaneous detection of T and B cell lymphopaenia, using an assay to measure surrogate markers of B cell production (kappa-deleting recombination excision circles, KREC) in addition to TREC[10]. This strategy enables identification of other forms of PID, including congenital B cell deficiency diseases (such as X-linked agammaglobulinaemia (XLA) and XLA-like autosomal recessive disorders, Nijmegen Breakage syndrome and late onset ADA deficiency)[10]. These conditions could otherwise be missed by a TREC-only assay. The combined TREC/KREC screening approach has not previously been assessed in a large, prospective population-based cohort.

Application of newborn screening tests beyond the newborn period

Until screening for PID becomes an integral component of newborn screening programs, patients will continue to present for clinical evaluation with suspected immunodeficiency diseases. There has been limited evaluation of the role of TREC and KREC assays beyond the newborn period in patients with suspected PID. KREC levels have been used to evaluate immune reconstitution after haematopoetic stem cell transplantation[11,12]. The role of TREC/KREC levels in the sub-classification of patients with CVID have also been evaluated[13-15]. However, the utility of TREC/KREC assays as an adjunctive diagnostic tool in the work-up of patients with suspected PID has not been evaluated in other contexts.

The need to expand current newborn screening technologies to enable detection of a higher number of primary immunodeficiency diseases

Currently available screening strategies enable the identification of infants with severe forms of PID manifested by T and/or B cell lymphopaenia which are detectable by TREC or TREC/KREC assays. However, these will capture only a fraction of the 320+ different forms of PID. Whilst it can be argued that SCID is an appropriate primary screening target (given its severity and the need for urgent treatment), it has been well established that late diagnosis of any form of PID is associated with poorer health outcomes for affected patients[16-18]. Various

strategies have been considered to enable the identification of other forms of PID that cannot be detected by TREC/KREC assays. These include protein-based assays to identify complement deficiencies and granulocyte disorders[19,20] and targeted sequencing, for example to determine *UNC13D* copy number variation to screen infants for familial haemophagocytic lymphohistiocytosis (FHL)[21].

Primary antibody deficiencies constitute the largest group of immunodeficiency disorders, and hypogammaglobulinaemia is also a presenting feature of several forms of PID, including SCID and other combined immunodeficiencies[1]. Due to the presence of maternal immunoglobulins in the neonatal circulation, it is not possible to accurately measure these proteins in infancy using standard methods such as nephelometry[20,22]. Hence, it is not yet possible to determine an infant's endogenous immunoglobulin production. It follows, then, that aside from those primary antibody deficiency disorders associated with low or absent KREC levels (XLA and XLA-like disease), there is no strategy by which infants can be screened for immunoglobulin deficiency disorders.

Given the advances in genomic medicine, the role of up-front next-generation sequencing (NGS) has been suggested as a potential newborn screening strategy. Rapid NGS has already been evaluated in neonatal and paediatric intensive care unit settings to facilitate early diagnosis and inform treatment decisions[23-26], and has already been described in the context of screening for PID[27,28]. This approach shows promise as a means by which to screen for a large number of diseases simultaneously.

The role of newborn screening in identifying disease susceptibility factors

In approaching the era of personalised medicine, it would be advantageous not only to screen individuals for disease states, but also to identify augmentable disease susceptibility factors, to enable the prevention of disease development or, at least, its progression. Knowledge of susceptibility factors enables the provision of patient-specific advice regarding the need for screening, therapy or prophylaxis. One such example is screening infants for polymorphisms in fucosyltransferase genes (*FUT2* and *FUT3*) which determine human blood group antigen expression and susceptibility to specific disease states, such as increased risk of rotavirus or norovirus infection[29-31].

1.2 Aims

This thesis aims to address several gaps in the literature regarding optimal strategies for newborn screening for PID. It also explores additional approaches which may enable detection of a wider range of conditions in the future. Specifically, this thesis aims to:

1. Determine the efficacy of a combined TREC/KREC screening approach for the identification of newborns with severe forms of PID manifested by T and/or B cell lymphopaenia
2. Determine the utility of screening assays beyond the newborn period, as part of the diagnostic evaluation of patients with suspected immunodeficiency
3. Establish a method for screening newborns for hypogammaglobulinaemia
4. Explore the role of novel genetic screening strategies to identify newborns with key disease susceptibility factors and facilitate the application of personalised medicine

1.3 Thesis Outline

This thesis is presented in publication format, and includes four published papers, one paper accepted for publication and one chapter presenting unpublished data from a small pilot study. Where relevant, supplementary figures and tables follow each publication.

The following chapter provides an extensive review of the literature, which explores the history and recent developments in the field of newborn screening for PID (Paper I). Gaps in the literature are also highlighted, several of which are addressed in the subsequent chapters of this thesis.

Materials and methods, including ethical considerations and laboratory techniques developed for this work are presented in Chapter 3. In Chapter 4, the findings of a prospective screening study are presented. In this study, almost 60,000 newborns were screened for PID manifested by T and/or B cell lymphopaenia (Paper II), providing evidence to support a combined TREC/KREC screening approach. Chapter 5 demonstrates the utility of newborn screening assays beyond infancy in the evaluation of patients with suspected PID by showing that KREC levels remain low or absent throughout life in patients with XLA (Paper III). In Chapter 6, early pilot data is presented from a small study evaluating a transcriptomic approach to screen children for hypogammaglobulinaemia using RNA extracted from dried blood spots. Chapter 7 provides an evaluation of the application of a genotyping-based method to screen newborns for polymorphisms in key genes which confer specific infectious disease susceptibility. This chapter also examines the implications of this screening strategy for provision of personalised medicine (Paper IV). In Chapter 8, the current status of newborn screening worldwide is presented, coupled with a forward-looking discussion of future testing strategies to expand current screening capabilities, including NGS (Paper V). The final chapter of this thesis provides a summary of the key findings arising from this research, together with a discussion of the clinical implications of these findings and directions for future research. Supplementary information and other details are provided as Appendices.

CHAPTER 2: LITERATURE REVIEW: NEWBORN SCREENING FOR PRIMARY IMMUNODEFICIENCY DISEASES

2.1 Introduction and Contextual Statement

This chapter presents a comprehensive review of the literature, which includes a discussion of the history of newborn screening, from its origins in the early 1960s. The review draws attention to currently available, and future strategies for newborn screening for immunodeficiency diseases.

2.2 Publication: Newborn Screening for Primary Immunodeficiency Diseases: History, Current and Future Practice

This chapter presents the first publication included in this thesis.

The paper entitled 'Newborn Screening for Primary Immunodeficiency Diseases**:** History, current and future practice' by Jovanka King and Lennart Hammarström was published in the peer reviewed journal, *Journal of Clinical Immunology*, in November 2017 (38:56, doi:10.1007/s10875-017-0455-x).

ARTICLE METRICS (AS AT 11 JANUARY 2019):

Journal Impact Factor: 3.382

Citations: 17

Altmetrics score: 2

Statement of Authorship

Title of Paper	Newborn Screening for Primary Immunodeficiency Diseases: History, Current and Future Practice
Publication Status	Published
Publication Details	Newborn Screening for Primary Immunodeficiency Diseases: History, Current and Future Practice. King JR, Hammarström L. J Clin Immunol. 2018 Jan;38(1):56-66. doi: 10.1007/s10875-017-0455-x. Epub 2017 Nov 8. PMID: 29116556

Principal Author

Name of Principal Author (Candidate)	Dr Jovanka King		
Contribution to the Paper	Literature review, writing of manuscript, production of figures and tables.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	11/4/18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Professor Lennart Hammarström		
Contribution to the Paper	Initiation of concept, oversight of manuscript content & structure, provision of critical review of manuscript.		
Signature		Date	2018-04-11

J Clin Immunol
https://doi.org/10.1007/s10875-017-0455-x

ORIGINAL ARTICLE

Newborn Screening for Primary Immunodeficiency Diseases: History, Current and Future Practice

Jovanka R. King[1,2] · **Lennart Hammarström**[1,3]

Received: 11 August 2017 / Accepted: 16 October 2017

Abstract The primary objective of population-based newborn screening is the early identification of asymptomatic infants with a range of severe diseases, for which effective treatment is available and where early diagnosis and intervention prevent serious sequelae. Primary immunodeficiency diseases (PID) are a heterogeneous group of inborn errors of immunity. Severe combined immunodeficiency (SCID) is one form of PID which is uniformly fatal without early, definitive therapy, and outcomes are significantly improved if infants are diagnosed and treated within the first few months of life. Screening for SCID using T cell receptor excision circle (TREC) analysis has been introduced in many countries worldwide. The utility of additional screening with kappa recombining excision circles (KREC) has also been described, enabling identification of infants with severe forms of PID manifested by T and B cell lymphopenia. Here, we review the early origins of newborn screening and the evolution of screening methodologies. We discuss current strategies employed in newborn screening programs for PID, including TREC and TREC/KREC-based screening, and consider the potential future role of protein-based assays, targeted sequencing, and next generation sequencing (NGS) technologies, including whole genome sequencing (WGS).

Keywords Newborn screening · Primary immunodeficiency diseases · TREC · KREC · Next-generation sequencing

This review is based on a keynote lecture given by Lennart Hammarström at the Clinical Immunology Society Meeting in Seattle on March 23rd, 2017

✉ Lennart Hammarström
lennart.hammastrom@ki.se

[1] Department of Clinical Immunology, Karolinska University Hospital Huddinge, SE-141 86 Stockholm, Sweden

[2] Department of Immunopathology, SA Pathology, Women's and Children's Hospital Campus; Robinson Research Institute and Discipline of Paediatrics, School of Medicine, University of Adelaide, North Adelaide, South Australia 5006, Australia

[3] BGI-Shenzhen, Shenzhen 518083, China

Introduction

Population-based newborn screening enables the early identification of asymptomatic infants with a range of severe diseases, for which effective treatment is available and where early diagnosis and intervention prevent serious sequelae. Primary immunodeficiency diseases (PID) are a heterogeneous group of inborn errors of immunity, the majority of which present in infancy and result in significant morbidity and mortality. Until recently, it was not possible to identify infants with PID prior to the onset of clinical symptoms, at which point they typically have complications of severe and protracted infection. Advances in technology have enabled identification of infants with severe forms of PID manifested by T and/or B cell lymphopenia. Here, we review the early origins of newborn screening and the evolution of screening methodologies. We review current strategies employed in newborn screening programs for PID, including T cell receptor excision circle (TREC) and kappa recombining excision circles (KREC)-based screening approaches, and discuss the potential future role of protein-based assays, targeted sequencing, and next generation sequencing (NGS) technologies.

The Early Origins of Newborn Screening

Phenylketonuria: Discovery, Treatment, and Screening

Phenylketonuria (PKU), also known as Følling's disease, was first identified in 1934 by the Norwegian biochemist and

Published online: 08 November 2017

physician Ivar Asbjörn Følling (1888–1973), who evaluated two siblings aged 4 and 7 years with developmental delay and an unusual urinary odor [1]. He postulated that these features might be linked and set about identifying a potential causative compound in their urine. As a result of a series of experiments, he hypothesized that defective phenylalanine metabolism was responsible for their symptoms. A survey of 430 patients in a nursing home and school for children with intellectual disability led to identification of eight further cases, including two sibling pairs, with further pedigree analyses suggesting an autosomal recessive inheritance pattern [1, 2]. Følling named the disease "imbecillitas phenylpyruvica," which in later years became known as "phenylketonuria," a term coined by English geneticist Lionel Penrose [2]. The biochemical defect was described by George Jervis in 1945 [3]. In 1953, Bickel et al. highlighted the beneficial effects of a phenylalanine-free diet in a child with PKU whose developmental progress markedly improved, but regressed upon cessation of the dietary restriction [4]. In 1956, Horner and Streamer reported marked improvement (but not complete reversal) of behavior and development in two children with PKU aged 4 and 4.5 years treated with a low-phenylalanine diet [5]. In comparison, when the diet was commenced at 8 weeks of age in the younger sibling of one of these children, the child developed normally [5], providing evidence for the necessity of early institution of the low-phenylalanine diet in infants with PKU.

Although infants with affected siblings could be identified and treated early, it became clear that all infants should be tested for PKU in the newborn period to prevent long-term sequelae [2]. In the late 1950s, Willard Centerwall developed a "diaper test" to screen newborns for PKU using a ferric chloride reaction on freshly wet diapers, which enabled early identification and treatment of many infants with PKU in California [6]. However, one limitation was the absence of the compound in the urine until the child was a few weeks old [2], prompting development of a blood testing strategy which could be carried out in the first few days of life. Robert Guthrie (1916–1995), an American microbiologist, described a method of specimen collection from newborns, where blood from a heel puncture was applied to filter paper, dried, and then subjected to testing [7]. This later became known as the "Guthrie card," and this method continues to be used in newborn screening programs worldwide. In 1963, Guthrie and Ada Susi described a method for detection of PKU in newborns based on the inhibition of growth of *Bacillus subtilis* by phenylalanine and associated compounds [7]. Small, circular punches taken from the dried blood spots (DBS) were placed on agar medium, and high levels of phenylalanine in PKU patients resulted in bacterial growth inhibition [7]. This marked the beginning of large-scale population screening in the USA, with the PKU screening program starting in Massachusetts in 1963, followed by rapid uptake in other states and in other countries around the world [8].

Recognition of the Important Role of Screening in Population Health

In 1968, James Maxwell Glover Wilson (1913–2006), Principal Medical Officer at the Ministry of Health, in London, England, and Gunnar Jungner (1914–1982), Chief of Clinical Chemistry at Sahlgren's Hospital in Gothenburg, Sweden, published their World Health Organization (WHO) report entitled "Principles and practice of screening for disease" [9]. This was, and remains to this day, a highly significant contribution to the public health and population screening literature. Their description of the principles underpinning screening practices and the "Wilson and Jungner Criteria" for disease inclusion in screening programs (Table 1) [9] are still highly relevant in guiding decision-making.

Beyond PKU: Expansion of Newborn Screening Programs

Following the successful implementation of newborn screening for PKU in several locations worldwide, attention was then given to expanding programs to screen for other types of metabolic disease and other conditions. In Sweden, screening for PKU commenced in 1965, followed by the addition of galactosemia (1967), congenital hypothyroidism (1980), congenital adrenal hyperplasia (1986), and biotinidase deficiency (2002). In 2010, an additional 19 disorders were added using tandem mass spectrometry and currently, 24 diseases are included in the national screening program.

Newborn Screening Methodologies

Through the work of these pioneers throughout history, newborn screening practices have evolved and expanded to include a wider number of screened diseases. Technological advances have enabled development of new assays, with improved sensitivity, specificity and capacity for automation [10]. From the first generation of PKU screening using ferric chloride reactions in neonatal diapers to Guthrie and Susi's bacterial inhibition assay, the next significant milestone in newborn screening methodology was the advent of tandem mass spectrometry (MS/MS). This technology enables identification of a compound in a biological sample based on the mass/charge ratio and provides an opportunity to screen for several compounds simultaneously, hence increasing the number of screened disorders [8]. This modality continues to be used in newborn screening programs around the world. Other techniques include spectrophotometry, fluorometry, and immunoassay [10].

Screening methodologies have subsequently expanded to include DNA-based testing strategies. A DNA-based screening program for glutaric academia type 1 was established in the Canadian provinces of Manitoba and north-western Ontario in 1998. Here, a high incidence of this disorder has been noted in a local indigenous population, attributable to a single homozygous

Table 1 Wilson and Jungner principles of early disease detection

1	The condition sought should be an important health problem.
2	There should be an accepted treatment for patients with recognized disease.
3	Facilities for diagnosis and treatment should be available.
4	There should be a recognizable latent or early symptomatic stage.
5	There should be a suitable test or examination.
6	The test should be acceptable to the population.
7	The natural history of the condition, including development from latent to declared disease, should be adequately understood.
8	There should be an agreed policy on whom to treat as patients.
9	The cost of case-finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditure on medical care as a whole.
10	Case-finding should be a continuing process and not a "once and for all" project.

Wilson and Jungner 1968 [9]

mutation in glutaryl-CoA-dehydrogenase [11]. Targeted sequencing of this mutation enabled identification of several affected infants, facilitating early institution of treatment [11]. Targeted genetic testing has also been included in newborn screening algorithms for cystic fibrosis, where an elevated immunoreactive trypsinogen measurement is followed by screening for a panel of *CFTR* (cystic fibrosis transmembrane conductance regulator) mutations [12]. A targeted genetic testing strategy has also been described for screening newborns for familial hemophagocytic lymphohistiocytosis (FHLH) due to *UNC13D* inversion mutations [13].

Newborn Screening Programs Worldwide

Therrell et al. recently published a comprehensive report on the status of newborn screening worldwide, which reflects the great variability in screening practices in different regions [14]. Most programs are structured to screen for a number of core disorders, along with secondary target disorders (typically organic and amino acidemias and fatty acid oxidation disorders) that are differential diagnoses of the core disorders [8]. As well as laboratory-based testing performed on dried blood spot samples, in many countries, newborns are also screened for hearing loss and cardiac disease using bedside assessment techniques [14]. There is currently a capacity to screen for over 50 different conditions, and diseases which are included in newborn screening programs or potential future inclusions are listed in Table 2. Decisions regarding disease inclusion at the local level should be guided by knowledge of the natural history of the disease, availability of treatment modalities, ability to decrease morbidity and mortality through screening and results of cost-effectiveness analyses [14].

Primary Immunodeficiency Diseases

Primary immunodeficiency diseases (PID) are a heterogeneous group of inborn errors of immunity, which were first recognized in the 1950s with the description by Ogden Bruton (1908–2003) of a young boy with recurrent infections and agammaglobulinemia [15]. Since this time, the broad clinical spectrum of PID has been recognized, and well over 300 different genetic mutations resulting in PID have been described to date [16]. Patients with PID typically present with a predisposition to infection, and delayed diagnosis results in significant complications and associated increased morbidity and mortality. PID present with a spectrum of clinical phenotypes and are caused by different pathophysiological mechanisms. They may be broadly classified as follows: immunodeficiencies affecting cellular and humoral immunity, combined immunodeficiencies with associated or syndromic features, predominant antibody deficiencies, immune dysregulatory diseases, congenital defects of phagocyte number or function, innate or intrinsic immune defects, autoinflammatory disorders, complement deficiencies, or PID phenocopies [16].

Severe combined immunodeficiency (SCID) is one of the most severe forms of PID and is manifested by a lack of T cells. B and NK cells may be variably absent depending on the molecular defect. This condition is an immunological emergency and requires prompt diagnosis and management. SCID is uniformly fatal without treatment. This condition came to public attention in the 1970s, when David Vetter (1971–1984), known as the "bubble boy," was diagnosed with SCID and immediately after birth, was placed in a protective sterile environment at the Houston Children's Hospital in Texas. Unfortunately, a matched donor for hematopoietic stem cell transplantation (HSCT) was not available, and David lived in the "bubble" until his death at age 13 years from EBV-associated lymphoma (https://primaryimmune.org/). David's case was an example of how early diagnosis and management of SCID augment clinical progress. Recent evidence suggests that patient outcomes are markedly improved if definitive therapy with HSCT is performed before the age of 3.5 months, prior to the onset of severe infections and other complications [17]. Realistically, this is

Table 2 Included conditions in newborn screening programs

Amino acid disorders
Phenylketonuria
Maple syrup urine disease
Homocystinuria
Citrullinemia type I
Argininsuccinic aciduria
Tyrosinemia I
Other secondary conditions (argininemia, citrullinemia type II, hypermethioninemia, benign hyperphenylalaninemia, biopterin defects, tyrosinemia type II and III)
Organic acid disorders
Methylmalonic acidemia (with or without homocystinuria)
Glutaric acidemia type I
Propionic acidemia
3-Methylcrotonyl-glycinuria
3-Hydroxy-3-methyl glutaric aciduria
Holocarboxylase synthase deficiency
β-Ketothiolase deficiency
Isovaleric acidemia
Other secondary conditions (malonic acidemia, isobutyrylglycinuria, 2-methylbutyrylglycinuria, 3-methylglutaconic acidurias, 2-methyl-3-hydroxybutyric acidurias)
Fatty acid β-oxidation disorders
Medium-chain acyl-CoA deficiency
Very long-chain acyl-CoA dehydrogenase deficiency
Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency
Trifunctional protein deficiency
Carnitine transport defect
Carnitine palmitoyltransferase I deficiency
Carnitine palmitoyltransferase II deficiency
Carnitine acylcarnitine translocase deficiency
Glutaric acidemia type II
Other secondary conditions (short-chain acyl-CoA dehydrogenase deficiency, medium-short-chain K-3-hydroxyacyl-CoA dehydrogenase deficiency, medium-chain ketoacyl-CoA thiolase deficiency, 2,4-dienoyl-CoA reductase deficiency)
Lysosomal storage disorders
Krabbe's disease
Pompe's disease
Fabry disease
Gaucher disease
Niemann pick disease
Mucopolysaccharidosis I
Mucopolysaccharidosis II
Other lysosomal disorders
Other
Congenital hypothyroidism
Human immunodeficiency virus (HIV)
Toxoplasmosis
Severe combined immunodeficiency (SCID)
X-linked adrenoleukodystrophy
Glucose-6-phosphate-dehydrogenase (G6PD) deficiency
Hemoglobinopathies
Sickle cell disease
Congenital adrenal hyperplasia
Classic galactosemia
Biotinidase deficiency
Cystic fibrosis
Duchenne muscular dystrophy
Galactokinase deficiency
Galactoepimerase deficiency
Point of care testing
Hearing loss
Congenital heart disease
Proposed future conditions
Fragile X syndrome
Ornithine transcarbamylase deficiency
Wilson disease
Guanidinoacetate methyltransferase deficiency

Villoria et al. [8], Clague and Thomas [10], Therrell et al. [14]

only achievable by early identification of infants with SCID through newborn screening programs.

History of Newborn Screening for Primary Immunodeficiency Diseases

Screening for Severe Combined Immunodeficiency (SCID)

Severe combined immunodeficiency (SCID) is an important health problem, for which the natural history is known and treatment is available, and as such, it meets the Wilson and Jungner criteria [9] and is a suitable candidate for population screening. SCID manifests with low or absent T lymphocytes, and thus screening newborns for T cell lymphopenia is an ideal strategy for identifying the disease. The first strategy proposed involved screening each newborn with a full blood count to determine the lymphocyte count [18], which was deemed to lack sensitivity. Subsequently, screening cord blood for T cell populations by flow cytometry was also considered [19]; however, given that this was likely to be time-consuming and expensive, other screening methods for the detection of T cell lymphopenia were considered.

T cell receptor excision circles (TREC) are small, circular pieces of episomal DNA which are formed during T cell receptor (TCR) rearrangement in naïve T cells and are thus surrogate markers for recent thymic emigrants. TREC were first visualized by electron microscopy as circular, extra-chromosomal DNA in mouse thymocytes in 1982 [20] and were later demonstrated to be the product of TCR rearrangement [21]. The TREC assay was developed by Douek et al. who demonstrated that TREC were specific to naïve T cells and described an age-related decline in healthy individuals and reduced levels in HIV infection [22]. TREC were noted to be stable, not prone to degradation, and did not replicate with subsequent cell division, making them an ideal marker

for naïve T cell production. In 2005, Kee Chan and Jennifer Puck first described application of the TREC assay for large-scale population screening for SCID and other forms of T cell lymphopenia [23]. The TREC assay was subsequently optimized, and the first state-wide SCID screening pilot study was commenced in Wisconsin in 2008, led by Jack Routes and James Verbsky [24]. Later that year, the first child with SCID who was identified by newborn screening was successfully transplanted (Jeffrey Model Foundation, http://www.info4pi.org). Subsequently, screening was implemented in Massachusetts, Louisiana, and New York in 2009, and California, Texas, and Pennsylvania in 2010. Screening for SCID is now routinely conducted or due to commence in the near future in the majority of states, including the District of Columbia, Navajo Nation and Puerto Rico, with 92% of American infants undergoing screening at the present time [25, https://primaryimmune.org/idf-advocacy-center/idf-scid-newborn-screening-campaign/].

Although identification of infants with SCID was the intended aim of TREC-based newborn screening programs, it became evident that in addition to this disorder, the assay would also identify infants with T cell lymphopenia due to other primary and secondary causes (Table 3). For example, low TREC levels have been detected in individuals with 22q deletion syndrome, CHARGE association, and Trisomy 21 [26]. In addition, infants with forms of PID other than SCID may have low TREC, for example in ataxia telangiectasia and combined immunodeficiency diseases (CID). Thus far in prospective pilot studies, many cases of CID without an identifiable molecular cause have been detected using the TREC assay [27, 28, 30] and these patients require clinical characterization and long-term follow-up.

In addition to TREC analysis, other SCID screening approaches have been proposed. In Italy, newborns are screened for SCID with the TREC assay, along with tandem mass spectrometry to identify ADA deficiency, which also enables identification of infants with delayed-onset ADA deficiency [31–33]. A similar approach has been described to identify patients with PNP deficiency [34]. A two-tiered testing approach using TREC analysis combined with IL-7 measurement has also been proposed as a means to increase the specificity of SCID screening [35]; however, this has not yet been optimized for application in large-scale screening programs [36].

Primary immunodeficiency diseases were previously thought to be rare entities, and the incidence of SCID was unknown. Prospective screening programs have since enabled the true incidence of SCID to be determined. Furthermore, screening has facilitated the identification and treatment of infants who would have otherwise died from complications of undiagnosed SCID. Based on studies in the USA where over 3 million infants were screened, the incidence of SCID was found to be much higher than expected at 1/58000 and the incidence of clinically relevant T cell lymphopenia was 1/7300 [27]. The annual live birth rate in the USA is approximately 4 million. Given that it was previously estimated that the incidence of SCID was 1/100000, screening has thereby increased the number of expected cases from approximately

Table 3 Disorders detectable by TREC and KREC screening

Low TREC levels	Low KREC levels
Severe combined immunodeficiency*	Severe combined immunodeficiency (T-B-)**
22q deletion syndrome	X-linked agammaglobulinemia (XLA)
Combined immunodeficiency	XLA-like disorders
Ataxia telangiectasia	Nijmegen breakage syndrome**
DOCK 8 deficiency	
EDA-ID	
Trisomy 21	
Trisomy 18	
Kabuki syndrome	
CHARGE syndrome	
Noonan syndrome	
Jacobsen syndrome	
Nijmegen breakage syndrome**	
Fryns syndrome	
Schimke immuno-osseous dysplasia	
Cartilage hair hypoplasia	
CLOVES	
ECC	
Rac2 defect	
Renpenning syndrome	
TAR	
Other cytogenetic abnormalities - Including 6p deletion, ring chromosome 14, ring chromosome 17, chromosome 17p duplication, 14q microdeletion	
Secondary causes	
Prematurity	Maternal immunosuppression
Congenital cardiac disease	Other maternal medications - Including ritodrine
Chylothorax	
Multiple congenital anomalies	
Gastrointestinal anomalies - Including gastroschisis	
Third space losses Vascular leakage Hydrops	
Neonatal leukemia	
Maternal autoimmune disease	
Maternal HIV infection	
Maternal immunosuppression	
Other maternal medications -Including ritodrine	

DOCK8, dedicator of cytokinesis 8; *CHARGE*, coloboma, heart defects, atresia choanae, growth retardation, genital abnormalities, ear abnormalities; *CLOVES*, congenital, lipomatous, overgrowth, vascular malformations, epidermal nevi, spinal/skeletal anomalies, and/or scoliosis; *ECC*, ectodermal dysplasia, ectrodactyly, and clefting; *TAR*, thrombocytopenia and absent radius; *HIV*, human immunodeficiency virus; *EDA-ID*, ectodermal dysplasia-associated immunodeficiency

*Excluding Zap70 deficiency, MHCII deficiency, and late-onset ADA deficiency

**Low TREC and KREC levels

Jyonuchi et al. [26], Kwan et al. [27], Chien et al. [28], Barbaro et al. [29]

40 to 69 annually, equating to an additional 29 cases each year identified and managed with potentially curative treatment. Given that PID constitute a large and heterogeneous group of genetic disorders which are individually rare entities, newborn screening offers additional benefits beyond individual patient care. Screening provides important opportunities to increase our knowledge of the clinical and pathophysiological spectrum of PID and gain additional experience in management of these conditions, particularly as affected infants are identified early in their disease course. Newborn screening thereby facilitates continued, collaborative research and progression of our knowledge in this field by enabling collation of rare and diverse cases and building national and international expertise.

Screening for Congenital B Cell Deficiency Disorders

Mutations in key genes which are essential for B cell ontogeny give rise to congenital B cell deficiency disorders including X-linked agammaglobulinemia (XLA) (resulting from a mutation in the *BTK* gene) and autosomal recessive XLA-like disorders. Patients have absent B cells, extremely low or undetectable immunoglobulin levels, and an increased susceptibility to severe infection with bacteria and other pathogens [16]. Like T cells, B cells also undergo rearrangement of the variable, diversity, and joining domains (V(D)J recombination) during development in order to produce unique B cell antigen receptors, and this process also yields episomal, circular DNA referred to as a kappa recombining excision circles (KREC). V(D)J recombination within the *IGK* locus results in a Vk-Jk coding joint, followed by rearrangement of the intron RSS and Kde elements with deletion of the Ck exon and enhancers. The coding joint remains present in the genome, whereas the KREC with the corresponding signal joint is excluded as a stable, circularized DNA fragment [37]. In 2007, van Zelm et al. described this process and developed a KREC assay using a PCR-based method [37]. They demonstrated that KREC levels reflected the replication history of B cells and had potential utility in assessing B cell recovery following HSCT and in the assessment of patients with antibody deficiency disorders such as common variable immunodeficiency (CVID) [37]. In 2011, Nakagawa et al. were the first to demonstrate the utility of the KREC assay in identifying newborns with B cell deficiency disorders, showing that signal joint KREC were absent in blood and Guthrie card specimens in patients with XLA [38].

Concurrent Screening for Severe Forms of Primary Immunodeficiency Manifested by T and B Cell Lymphopenia

Given the potential utility of the KREC assay in newborn screening for PID, a multiplexed TREC/KREC assay has been described, which enables simultaneous identification of infants with severe forms of PID manifested by T and/or B cell lymphopenia (Table 3 and 4) [40]. This approach has since been adopted in newborn screening pilot studies in Sweden and Spain [29, 41]. Multiplexed TREC/KREC assays offer many advantages over TREC screening alone, as well as identification of congenital B cell defects; it enables identification of individuals with different forms of PID which might be missed by a TREC-alone assay, including late-onset ADA deficiency, some cases of Nijmegen breakage syndrome and other selected disorders [40].

Limitations of Current Screening Strategies for Primary Immunodeficiency

It has been established that although TREC screening will identify the majority of infants with SCID, cases where the molecular defect lies downstream of T cell receptor rearrangement will not be detected. This includes Zap70 deficiency, MHC Class II deficiency, and some cases of delayed ADA deficiency [42–47]. Defects of T cell function despite a quantitatively normal T cell number will also not be detected by the TREC assay.

The Current Status of Newborn Screening for Primary Immunodeficiency Diseases Worldwide

Screening programs for PID have been instituted in many regions. TREC-based PID screening programs have been established in the majority of American states (including the Navajo region, District of Columbia, and Puerto Rico), Taiwan, Israel, Qatar, and several Canadian regions, and are due to commence in New Zealand in the near future [48]. Screening programs utilizing TREC-only, TREC/KREC, or TREC/ADA strategies have also been evaluated in pilot studies in Italy, Sweden, Germany, The Netherlands, Japan, France, Spain, Norway, France, the UK, Turkey, Slovenia, Saudi Arabia, Iran, Iceland, Denmark, and Brazil, and many more regions have applications in progress to commence screening programs [48].

The Future of Newborn Screening for Primary Immunodeficiency Diseases

Screening for Complement and Granulocyte Disorders Using Protein-Based Methods

Complement proteins are an important component of the innate immune system, and deficiencies in complement give rise to a variable clinical phenotype, including autoimmune disease, renal disease, and a susceptibility to specific bacterial infections which frequently result in life-threatening infection [16]. Disorders of granulocyte number and function are an important group of disorders, with affected individuals

Table 4 Results of prospective newborn screening programs for primary immunodeficiency

Region	Screening period	Screening strategy	Number of newborns screened	Primary immunodeficiency cases identified	References
USA (10 states + Navajo region)	January 2008–July 2013 (5.5 years)	TREC	3,030,083	SCID (*n* = 52) - Typical SCID (*n* = 42) - *IL2RG* (*n* = 9) - *IL7RA* (*n* = 6) - *ADA* (*n* = 5) - *RAG1* (*n* = 4) - *JAK3* (*n* = 3) - *DCLRE1C* (*n* = 1) - *RAG2* (*n* = 1) - *CD3D* (*n* = 1) - *TC7A* (*n* = 1) - Pallister-Killian syndrome; tetrasomy 12p (*n* = 1) - Molecular defect unknown (*n* = 6) - Genetic testing incomplete (*n* = 4) - Leaky SCID (*n* = 10) - *RAG1* (*n* = 4) - *RMRP* (*n* = 2) - *IL2RG* (*n* = 1) - *DCLRE1C* (*n* = 1) - Molecular defect unknown (*n* = 2)	Kwan et al. 2014 [27]
Taiwan	2010–2017 (78 months)	TREC	920,398	SCID (*n* = 7) - *IL2RG* (*n* = 3) - *RAG1* (*n* = 1) - Molecular defect unknown (*n* = 3) SCID variant, molecular defect unknown (*n* = 8) EDA-HT (*n* = 1)	Chien et al. 2017 [28]
Sweden (Stockholm county)	15 November 2013–14 November (3 years)	TREC/KREC	89,462	SCID (*n* = 2) - Artemis deficiency) (*n* = 1) - *ADA* deficiency (*n* = 1) Ataxia telangiectasia (*n* = 1) CID, molecular defect unknown (*n* = 2)	Barbaro et al. 2016 [29] Zetterström et al. 2017 [30]
Israel	1 October 2015–30 April 2017 (18 months)	TREC	290,864	SCID (*n* = 13) - Typical SCID (*n* = 10) - *DCLRE1C* (*n* = 3) - *IL7RA* (*n* = 2) - *RMRP* (*n* = 1) - Ligase 4 deficiency (*n* = 1) - Complete DiGeorge Syndrome (*n* = 1) - Molecular defect unknown (*n* = 2) - Leaky SCID (*n* = 3) - *DCLRE1C* (*n* = 2) - MHC2 deficiency/*RFX5* (*n* = 1) Undefined PID (*n* = 6)	Rechavi et al. 2017 [39] Rechavi et al. (personal communation)

TREC T cell receptor excision circles, *KREC* kappa recombining excision circles, *SCID* severe combined immunodeficiency, *CID* combined immunodeficiency, *EDA-HT* X-linked recessive anhidrotic ectodermal dysplasia-associated immunodeficiency

typically presenting with severe bacterial or fungal infections and other features such as colitis [16]. Atypical presentations frequently result in delayed diagnosis and treatment. Patients with complement or granulocyte disorders will not be

identified using current TREC or TREC/KREC-based methodologies, which identify PID manifesting with T and/or B cell lymphopenia, respectively. As such, protein-based screening methodologies have been proposed as a means by which to identify infants with complement and granulocyte disorders. Specific complement proteins, including C2 and C3, can be eluted from DBS and quantified, enabling identification of infants with low or undetectable protein levels at birth [49, 50], facilitating early intervention with prophylactic measures to prevent potentially catastrophic outcomes. It has also recently been demonstrated that complement deficiency can be identified using a whole genome sequencing (WGS)-based newborn screening strategy [51].

Screening for Familial Hemophagocytic Lymphohistiocytosis

Targeted DNA sequencing has previously been employed as a screening strategy for selected diseases, such as glutaric acidemia type I and cystic fibrosis. This approach has also been described as a potential method by which infants with familial hemophagocytic lymphohistiocytosis (FHLH) due to mutations in *UNC13D* may be identified [13]. Fifty percent of FHLH cases in Scandinavia are due to homozygous *UNC13D* inversion mutations, and it has been demonstrated that a reduction in the wild type gene copy numbers is an effective way to screen for affected individuals [13].

The Role of Next Generation Sequencing in Newborn Screening for PID

Primary immunodeficiency diseases are a heterogeneous group of disorders, which differ in terms of clinical phenotype, laboratory findings, and underlying molecular abnormalities. There are currently over 300 different genetic mutations associated with PID [16], and this number continues to increase. As such, there is no single test which can reliably identify all infants with PID at birth. Rapid advances in genomic medicine have resulted in increased availability and reduced costs of next-generation sequencing (NGS), and whole exome sequencing (WES) and whole genome sequencing (WGS) have an established role in diagnostic medicine. Previously, the time taken from sample collection to receipt of results of NGS-based studies has been prolonged, often taking weeks or months. However, "rapid" NGS has been described in the setting of critically ill infants in the pediatric intensive care unit (PICU) and neonatal intensive care unit (NICU) setting, where a 26 hour turnaround time has been achieved, and results have impacted upon patient care [52–54]. In one cohort of 35 acutely unwell infants, 20 were diagnosed with a genetic disease using rapid WGS, 13 of whom had de novo mutations identified, and the diagnosis directly influenced management decisions (including specific management or palliation) in 10 cases [52]. It follows then, that in addition to diagnostic medicine, NGS is likely to have a future role in newborn screening for PID and other conditions.

The National Institutes of Health is currently evaluating the role of up-front NGS in newborn screening as part of the NSIGHT (Newborn Sequencing in Genomic Medicine and Public Health) project [55]. Whole-exome-based newborn screening for currently screened and additional disorders will be evaluated, along with the experience of parents and clinicians in the exchange and utility of genomic information. The ethical, legal, and social implications will also be explored [55]. In addition, rapid NGS in the NICU setting will be further evaluated [55].

Recently, Pavey et al. used WGS to screen 1349 newborn and parent trios for variants in 329 known PID-associated genes [51]. Applying a genotype-first pipeline, pathogenic or likely pathogenic mutations were identified in 396 infants; however, only one was found to have a genomically predicted PID (complement component C9 deficiency). A phenotype-first approach resulted in identification of 29 infants in the cohort who were potentially immunodeficient based on clinical features; however, no mutations were identified in the interrogated PID genes. Pathogenic mutations were identified in other (non-PID associated) genes in three of the children [51]. Lucarelli et al. also recently described a WGS-based screening strategy for cystic fibrosis, involving interrogation of a panel of 188 *CFTR* mutations [56]. Bodian et al. also evaluated up-front WGS as a screening strategy to evaluate 1696 newborns for variants in 163 genes which are implicated in diseases which are currently screened for in the USA, demonstrating that WGS was complementary to conventional newborn screening and gave fewer false positive results, resolved inconclusive findings and provided more precise diagnostic information compared to conventional techniques [57].

Applying a WGS-based approach to newborn screening represents a change in paradigm, and there are many factors which must be taken into consideration before adopting this approach in population-based screening programs [48, 58]. Firstly, candidate diseases and target genes for evaluation should be identified. In the case of PID, an ideal starting point would be establishing a panel of the currently identified 300+ PID-associated genes, which would need to be updated as new genes are described. Care must be taken to screen only for diseases with an established genotype-phenotype correlation. Robust and cost-effective testing systems must be established. The tests must be appropriately sensitive and specific, with an agreeable turnaround time and data should be analyzed and managed appropriately. An appropriate pipeline should be established to manage abnormal results, including confirmatory and second-tier testing and seamless integration with clinical services. The cost-effectiveness of this screening approach must also be formally assessed. Ethical, legal, and social implications are major considerations, including issues pertaining to consent, biobanking of genetic material, and data

and implications of genetic findings for other family members. A plan must also be in place for management of variants of unknown significance, including mutations in genes known to cause debilitating or lifespan-reducing disease for which there is no known treatment [48]. A screening approach using up-front WGS must be evaluated in large, prospective trials prior to adopting this strategy in population-based newborn screening programs. We support the consideration of WGS as an up-front screening method.

Management of PID is variable and is dependent on the disease, clinical phenotype, and molecular defect. In the case of SCID, HSCT or gene therapy is currently available curative therapies. Advances in CRISPR-Cas based genome editing have led to interest in potential therapeutic applications of this modality. Scott and Zhang recently highlighted that prior to institution of CRISPR-based therapy, patients should be screened using WGS to ensure safety and reduction of off-target effects [59]. The early identification of a specific PID-associated mutation as a result of newborn screening by NGS will enable timely initiation of the aforementioned therapies, along with other targeted, personalized treatment options [60].

Prenatal Screening

Newborn screening enables identification of infants with key disorders requiring early intervention soon after birth. Prenatal screening for disorders such as Trisomy 21 and neural tube defects is well established in obstetric practice, along with prenatal diagnostics in the case of a high risk of a specific genetic disorder based upon positive family history or other factors. However, this has typically required invasive procedures which carry associated risks, such as amniocentesis and chorionic villus sampling. Technological advances have resulted in the ability to detect fetal DNA in maternal plasma, and Lo et al. demonstrated that massively parallel sequencing (MPS) enables detection of fetal aneuploidies such as trisomies 21, 13, and 18 [61]. In addition to chromosomal aneuploidy, this technique also enables detection of sub-chromosomal deletions and duplications including 22q11 (DiGeorge Syndrome) and 5p (Cri-du-chat Syndrome) deletions and deletions associated with Prader-Willi and Angelman Syndromes. Monogenic traits and disorders such as RhD status and *FGFR3* mutations which give rise to achondroplasia can also be identified. Targeted or whole genome sequencing of the fetus is also possible, along with sequencing of the fetal transcriptome and methylome, enabling identification of a wide range of disorders [62]. Prenatal screening technologies may well also become part of the changing landscape of screening practices for PID and other disorders in the future.

Conclusion

Newborn screening practices worldwide have evolved significantly since the discovery of PKU in 1934, with rapid development of new and improved assays to enable identification of a wider range of conditions in infants and facilitating early diagnosis and treatment, and improving clinical outcomes. Primary immunodeficiency diseases are a heterogeneous group of disorders, and no single assay at the present time will identify all forms of PID, necessitating a challenge of current newborn screening paradigms. In the genomic era, it is likely that this will involve up-front next generation sequencing, including whole exome sequencing and ultimately, whole genome sequencing. This approach must be evaluated in large, prospective trials prior to adopting this strategy in population-based newborn screening programs.

Acknowledgements Dr. Jovanka R. King is the recipient of the Mike and Carole Ralston Traveling Fellowship 2016 from the RCPA Foundation.

Authorship Contributions Jovanka King and Lennart Hammarström wrote the paper.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Centerwall WR, Centerwall SA. Phenylketonuria (FOLLING's disease). The story of its discovery. J Hist Med Allied Sci. 1961;16:292–6.
2. Centerwall SA, Centerwall WR. The discovery of phenylketonuria: the story of a young couple, two retarded children, and a scientist. Pediatrics. 2000;105(1 Pt 1):89–103.
3. Jervis GA. Studies On Phenylpyruvic Oligophrenia: The position of the metabolic error. J Biol Chem. 1947;169(3):651–6.
4. Bickel H, Gerrard J, Hickmans E. Preliminary communication. Lancet. 1953;262(6790):812–3.
5. Horner FA, Streamer CW. Effect of a phenylalanine-restricted diet on patients with phenylketonuria; clinical observations in three cases. J Am Med Assoc. 1956;161(17):1628–30.
6. Centerwall WR. Phenylketonuria. J Am Med Assoc. 1957;165(4):392.
7. Guthrie R, Susi A. A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. Pediatrics. 1963;32:338–43.

8. Villoria JG, Pajares S, López RM, Marin JL, Ribes A. Neonatal screening for inherited metabolic diseases in 2016. Semin Pediatr Neurol. 2016;23(4):257–72.
9. Wilson J, Jungner G. The principles and practice of screening for disease. Geneva: World Health Organization; 1968.
10. Clague A, Thomas A. Neonatal biochemical screening for disease. Clin Chim Acta. 2002;315(1):99–110.
11. Greenberg CR, Prasad AN, Dilling LA, Thompson JR, Haworth JC, Martin B, et al. Outcome of the first 3-years of a DNA-based neonatal screening program for glutaric acidemia type 1 in Manitoba and northwestern Ontario, Canada. Mol Genet Metab. 2002;75(1):70–8.
12. Rosenfeld M, Sontag MK, Ren CL. Cystic fibrosis diagnosis and newborn screening. Pediatr Clin N Am. 2016;63(4):599–615.
13. Borte S, Meeths M, Liebscher I, Krist K, Nordenskjold M, Hammarstrom L, et al. Combined newborn screening for familial hemophagocytic lymphohistiocytosis and severe T- and B-cell immunodeficiencies. J Allergy Clin Immunol. 2014;134(1):226–8.
14. Therrell BL, Padilla CD, Loeber JG, Kneisser I, Saadallah A, Borrajo GJC, et al. Current status of newborn screening worldwide: 2015. Semin Perinatol. 2015;39(3):171–87.
15. Bruton OC. Agammaglobulinemia. Pediatrics. 1952;9(6):722–8.
16. Picard C, Al-Herz W, Bousfiha A, Casanova JL, Chatila T, Conley ME, et al. Primary immunodeficiency diseases: an update on the classification from the International Union of Immunological Societies Expert Committee for primary immunodeficiency 2015. J Clin Immunol. 2015;35(8):696–726.
17. Pai SY, Logan BR, Griffith LM, Buckley RH, Parrott RE, Dvorak CC, et al. Transplantation outcomes for severe combined immunodeficiency, 2000-2009. N Engl J Med. 2014;371(5):434–46.
18. Buckley RH, Schiff RI, Schiff SE, Markert ML, Williams LW, Harville TO, et al. Human severe combined immunodeficiency: genetic, phenotypic, and functional diversity in one hundred eight infants. J Pediatr. 1997;130(3):378–87.
19. Collier F, Tang M, Ponsonby AL, Vuillermin P. Flow cytometric assessment of cord blood as an alternative strategy for population-based screening of severe combined immunodeficiency. J Allergy Clin Immunol. 2013;131(4):1251–2.
20. Yamagishi H, Kunisada T, Tsuda T. Small circular DNA complexes in eucaryotic cells. Plasmid. 1982;8(3):299–306.
21. Fujimoto S, Yamagishi H. Isolation of an excision product of T-cell receptor alpha-chain gene rearrangements. Nature. 1987;327(6119):242–3.
22. Douek DC, McFarland RD, Keiser PH, Gage EA, Massey JM, Haynes BF, et al. Changes in thymic function with age and during the treatment of HIV infection. Nature. 1998;396(6712):690–5.
23. Chan K, Puck JM. Development of population-based newborn screening for severe combined immunodeficiency. J Allergy Clin Immunol. 2005;115(2):391–8.
24. Routes JM, Grossman WJ, Verbsky J, Laessig RH, Hoffman GL, Brokopp CD, et al. Statewide newborn screening for severe T-cell lymphopenia. JAMA. 2009;302(22):2465–70.
25. Dorsey M, Puck J. Newborn screening for severe combined immunodeficiency in the US: current status and approach to management. Int J Neonatal Screen. 2017;3(2):15.
26. Jyonouchi S, Jongco AM, Puck J, Sullivan KE. Immunodeficiencies associated with abnormal newborn screening for T cell and B cell lymphopenia. J Clin Immunol. 2017;37(4):363–74.
27. Kwan A, Abraham RS, Currier R, Brower A, Andruszewski K, Abbott JK, et al. Newborn screening for severe combined immunodeficiency in 11 screening programs in the United States. JAMA. 2014;312(7):729–38.
28. Chien Y-H, Yu H-H, Lee N-C, Ho H-C, Kao S-M, Lu M-Y, et al. Newborn screening for severe combined immunodeficiency in Taiwan. Int J Neonatal Screen. 2017;3(3):16.
29. Barbaro M, Ohlsson A, Borte S, Jonsson S, Zetterström RH, King J, et al. Newborn screening for severe primary immunodeficiency diseases in Sweden-a 2-year pilot TREC and KREC screening study. J Clin Immunol. 2017;37(1):51–60.
30. Zetterström R, Barbaro M, Ohlsson A, Borte S, Jonsson S, Winiarski J, et al. Newborn screening for primary immune deficiencies with a TREC/KREC/ACTB triplex assay—a three-year pilot study in Sweden. Int J Neonatal Screen. 2017;3(2):11.
31. la Marca G, Giocaliere E, Malvagia S, Funghini S, Ombrone D, Della Bona ML, et al. The inclusion of ADA-SCID in expanded newborn screening by tandem mass spectrometry. J Pharm Biomed Anal. 2014;88:201–6.
32. Azzari C, la Marca G, Resti M. Neonatal screening for severe combined immunodeficiency caused by an adenosine deaminase defect: a reliable and inexpensive method using tandem mass spectrometry. J Allergy Clin Immunol. 2011;127(6):1394–9.
33. la Marca G, Canessa C, Giocaliere E, Romano F, Duse M, Malvagia S, et al. Tandem mass spectrometry, but not T-cell receptor excision circle analysis, identifies newborns with late-onset adenosine deaminase deficiency. J Allergy Clin Immunol. 2013;131(6):1604–10.
34. la Marca G, Canessa C, Giocaliere E, Romano F, Malvagia S, Funghini S, et al. Diagnosis of immunodeficiency caused by a purine nucleoside phosphorylase defect by using tandem mass spectrometry on dried blood spots. J Allergy Clin Immunol. 2014;134(1):155–9.
35. McGhee SA, Stiehm ER, Cowan M, Krogstad P, McCabe ER. Two-tiered universal newborn screening strategy for severe combined immunodeficiency. Mol Genet Metab. 2005;86(4):427–30.
36. Bausch-Jurken M, Verbsky J, Routes J. Newborn screening for severe combined immunodeficiency-a history of the TREC assay. Int J Neonatal Screen. 2017;3(2):14.
37. van Zelm MC, Szczepanski T, van der Burg M, van Dongen JJ. Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. J Exp Med. 2007;204.
38. Nakagawa N, Imai K, Kanegane H, Sato H, Yamada M, Kondoh K, et al. Quantification of kappa-deleting recombination excision circles in Guthrie cards for the identification of early B-cell maturation defects. J Allergy Clin Immunol. 2011;128(1):223–5. e2
39. Rechavi E, Lev A, Saraf-Levy T, Etzioni A, Almashanu S, Somech R. Newborn screening for severe combined immunodeficiency in Israel. Int J Neonatal Screen. 2017;3(2):13.
40. Borte S, von Döbeln U, Fasth A, Wang N, Janzi M, Winiarski J, et al. Neonatal screening for severe primary immunodeficiency diseases using high-throughput triplex real-time PCR. Blood. 2012;119(11):2552–5.
41. de Felipe B, Olbrich P, Lucenas JM, Delgado-Pecellin C, Pavon-Delgado A, Marquez J, et al. Prospective neonatal screening for severe T- and B-lymphocyte deficiencies in Seville. Pediatr Allergy Immunol. 2016;27(1):70–7.
42. Grazioli S, Bennett M, Hildebrand KJ, Vallance H, Turvey SE, Junker AK. Limitation of TREC-based newborn screening for ZAP70 severe combined immunodeficiency. Clin Immunol. 2014;153(1):209–10.
43. Hauck F, Blumenthal B, Fuchs S, Lenoir C, Martin E, Speckmann C, et al. SYK expression endows human ZAP70-deficient CD8 T cells with residual TCR signaling. Clin Immunol. 2015;161(2):103–9.
44. Lev A, Simon AJ, Broides A, Levi J, Garty BZ, Rosenthal E, et al. Thymic function in MHC class II-deficient patients. J Allergy Clin Immunol. 2013;131(3):831–9.
45. Kuo CY, Chase J, Garcia Lloret M, Stiehm ER, Moore T, Aguilera MJ, et al. Newborn screening for severe combined immunodeficiency does not identify bare lymphocyte syndrome. J Allergy Clin Immunol. 2013;131(6):1693–5.

46. Lyttle A, Roifman C, Dadi H, Wright N, Kavadas F. MHC class II deficiency in the dene native population: a case report highlighting pitfalls in diagnosis and treatment. Allergy Asthma Clin Immunol. 2014;10(supplement 1):A1.
47. Speckmann C, Neumann C, Borte S, la Marca G, Sass JO, Wiech E, et al. Delayed-onset adenosine deaminase deficiency: strategies for an early diagnosis. J Allergy Clin Immunol. 2012;130(4):991–4.
48. King J, Ludvigsson J, Hammarström L. Newborn screening for primary immunodeficiency diseases: the past, the present and the future. Int J Neonatal Screening. 2017;3(3):19.
49. Janzi M, Sjöberg R, Wan J, Fischler B, von Döbeln U, Isaac L, et al. Screening for C3 deficiency in newborns using microarrays. PLoS One. 2009;4(4):e5321.
50. Hamsten C, Skattum L, Truedsson L, von Döbeln U, Uhlén M, Schwenk JM, et al. Heat differentiated complement factor profiling. J Proteome. 2015;126:155–62.
51. Pavey AR, Bodian DL, Vilboux T, Khromykh A, Hauser NS, Huddleston K, et al. Utilization of genomic sequencing for population screening of immunodeficiencies in the newborn. Genet Med. 2017.
52. Willig LK, Petrikin JE, Smith LD, Saunders CJ, Thiffault I, Miller NA, et al. Whole-genome sequencing for identification of Mendelian disorders in critically ill infants: a retrospective analysis of diagnostic and clinical findings. Lancet Respir Med. 2015;3(5):377–87.
53. Kingsmore SF. Newborn testing and screening by whole-genome sequencing. Genet Med. 2016;18(3):214–6.
54. Smith LD, Willig LK, Kingsmore SF. Whole-exome sequencing and whole-genome sequencing in critically ill neonates suspected to have single-gene disorders. Cold Spring Harb Perspect Med. 2015;6(2):a023168.
55. Berg JS, Agrawal PB, Bailey DB, Beggs AH, Brenner SE, Brower AM, et al. Newborn sequencing in genomic medicine and public health. Pediatrics. 2017;139(2).
56. Lucarelli M, Porcaro L, Biffignandi A, Costantino L, Giannone V, Alberti L, et al. A new targeted CFTR mutation panel based on next-generation sequencing technology. J Mol Diagn. 2017.
57. Bodian DL, Klein E, Iyer RK, Wong WS, Kothiyal P, Stauffer D, et al. Utility of whole-genome sequencing for detection of newborn screening disorders in a population cohort of 1,696 neonates. Genet Med. 2016;18(3):221–30.
58. Friedman JM, Cornel MC, Goldenberg AJ, Lister KJ, Sénécal K, Vears DF, et al. Genomic newborn screening: public health policy considerations and recommendations. BMC Med Genet. 2017;10(1):9.
59. Scott DA, Zhang F. Implications of human genetic variation in CRISPR-based therapeutic genome editing. Nat Med. 2017.
60. Seleman M, Hoyos-Bachiloglu R, Geha RS, Chou J. Uses of next-generation sequencing technologies for the diagnosis of primary immunodeficiencies. Front Immunol. 2017;8(847).
61. Lo YM, Lun FM, Chan KC, Tsui NB, Chong KC, Lau TK, et al. Digital PCR for the molecular detection of fetal chromosomal aneuploidy. Proc Natl Acad Sci U S A. 2007;104(32):13116–21.
62. Wong FC, Lo YM. Prenatal diagnosis innovation: genome sequencing of maternal plasma. Annu Rev Med. 2016;67:419–32.

CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction and Contextual Statement

This chapter presents an overview of the materials and methods used to conduct the work included in this thesis. In this chapter, ethical considerations, patient and sample recruitment procedures and a summary of the laboratory techniques utilised, are described.

3.2 Ethical Considerations

All research included in this thesis was performed in accordance with the ethical standards of the Regional Ethical Board in Stockholm (Regional Ethical Committee, Karolinska Institutet), and the Women's and Children's Health Network Human Research Ethics Committee and Research Governance Committee. All research was performed in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Ethics approval was sought and obtained from the local ethical committees in each jurisdiction where the research was conducted:

- Regional Ethical Committee, Karolinska Institutet
    - 2013/414-31/4
    - 2016/189-31/2
- Women's and Children's Health Network Human Research Ethics Committee and Research Governance Committee
    - HREC/16/WCHN/164
    - SSA/16/WCHN/192

3.3 Participant and Sample Recruitment

Research participants were patients recruited from clinical immunology departments at three clinical centres in Sweden and one centre in South Australia. Healthy control participants were also recruited from each site. Written, informed consent was obtained from all participants recruited into the studies. Anonymised biological samples (i.e., dried blood spot specimens) were used in accordance with the standing Regional Ethical Committee and Karolinska Institutet / Women's and Children's Health Network policies permitting the use of such samples for research purposes.

3.3.1 Paper II (Chapter 4): TREC/KREC newborn screening study

Dried blood spot (DBS) samples from 58,834 infants recruited to the TREC/KREC screening study were obtained and processed at the Center for Inherited Metabolic Diseases (CMMS), Karolinska University Hospital, Stockholm.

3.3.2 Paper III (Chapter 5): KREC in agammaglobulinaemia study

Thirteen patients with agammaglobulinaemia were recruited from the clinical immunology departments in three clinical centres in Sweden. Whole blood and DBS specimens were collected from each patient. Where possible, each patient's original DBS, collected at birth, was accessed from archives at CMMS. Stored DNA samples, extracted at the time of diagnosis for each patient, were collected from the Clinical Research Centre at the Karolinska University Hospital. Anonymised, time-controlled specimens were also obtained from CMMS.

3.3.3 Unpublished paper (Chapter 6): Transcriptomic approach to screening for hypogammaglobulinaemia study

Nineteen patients and 13 healthy control subjects were recruited from a single South Australian clinical immunology department. Anonymised newborn DBS samples were provided by the South Australian Neonatal Screening Laboratory.

3.3.4 Paper IV (Chapter 7): Fucosyltransferase polymorphism screening study

Anonymised DBS specimens were obtained from 520 newborns. These were sourced from CMMS. Five hundred and twenty anonymised DBS specimens were obtained from CMMS, as described for Chapter 5 above.

3.4 DNA Extraction from Dried Blood Spots

DNA was extracted from DBS samples using the following method, optimised for a 96-well plate format. One 3.2mm punch was taken from each DBS sample, and placed in a 96-well plate. Ninety μL of Qiagen Generation DNA Purification Solution 1 was added to each well. The plate was centrifuged briefly, then incubated for 15 minutes at room temperature. The plate was then centrifuged at 2204 g for 5 minutes. The supernatant was removed from the wells. This procedure was repeated once, before addition of 90μL of sterile water to each well. The plate was briefly centrifuged before removal of the supernatant. Twenty μL of DNA Elution Solution 2 including 1μL/mL of yeast tRNA was added to each well before brief vortex mixing and centrifugation. The plate was then incubated in a thermal cycler (SimpliAmp Thermal Cycler, Thermo Fisher Scientific, MA, USA) at 99^0C for 30 minutes. The plate was briefly centrifuged before transfer of the eluate into another 96-well plate.

3.5 DNA Extraction from Whole Blood

DNA was extracted from whole blood samples using the salting-out method. Briefly, 10mL of blood and 40mL of lysis buffer were placed in a Falcon tube and gently inverted, followed by incubation at 37^0C for 10 minutes. The tube was centrifuged at 1500 rpm for 10 minutes at room temperature. The supernatant was discarded, 25mL of lysis buffer was added and gently shaken to dissolve the pellet. The tube was centrifuged at 1500 rpm for 10 minutes at room temperature and the supernatant was discarded. Ten mL of DNA buffer and 100μL of DNA Proteinase K was added to the pellet and incubated for 12 hours at 50^0C. Five mL of 6M sodium chloride was added and mixed for 30 minutes on a mixing table, before centrifugation at 4000 rpm (with no brake) for 10 minutes at room temperature. The supernatant was transferred to a new tube and the centrifugation step repeated. Twenty mL of 99% ethanol was added and mixed by hand, before centrifugation at 4000 rpm for 5 minutes at room temperature. The ethanol was discarded. Five mL of 70% ethanol was added, before centrifugation at 4000 rpm for 5 minutes at room temperature. The pellet loosened and transferred to an Eppendorf tube, which was then centrifuged at 13,000 rpm for 3 minutes. The ethanol was removed, and the tube dried for 10 minutes. One hundred - 800μL of TE buffer was added to the pellet (depending on pellet size) and incubated at room temperature for 48 hours.

3.6 TREC/KREC/ACTB Quantitation by qPCR

Eight μL of DNA extracted from the DBS specimens, or 40ng of DNA extracted from whole blood, was added to 10μL of TaqMan Gene Expression Master Mix and 2μL of TREC/KREC/ACTB primer mix (Applied Biosystems, California, USA) in a 96-well plate. The plate was run on an Applied Biosystems Quantstudio 7500 qPCR instrument, under the following conditions: hold at 50^0C for 2 minutes, heating cycle at 95^0C for 20 seconds, followed by 45 cycles of 30 seconds at 95^0C and 30 seconds at 60^0C.

3.7 RNA Extraction from Dried Blood Spots & cDNA Synthesis

Two 3.2mm punches were removed from each DBS sample for each specimen. RNA was extracted from the DBS punches using a method we developed. Briefly, the DBS punches were eluted in 200μL of rapid RNA extraction solution (Ambion, Applied Biosystems, MA, USA), and incubated at room temperature on a plate shaker for 15 minutes. The eluate was removed, and RNA and DNA was extracted using the QiaAMP MinElute Virus Spin Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesised using the Bio-Rad cDNA iScript synthesis kit (Bio-Rad, California, USA). Twenty nanograms of RNA was added to 4μL of 5X iScript Reaction Mix, 1μL of iScript Reverse Transcriptase and nuclease-free water to give a final volume of 20μL. Thermal cycler conditions were as follows:

Priming 25^{0}C for 5 minutes, reverse transcription 46^{0}C for 20 minutes, RT inactivation 95^{0}C for 1 minute, hold 4^{0}C for 20 minutes.

3.8 Gene Expression Analysis

Five microlitres of the cDNA product, diluted 1:5, was added to 10μL of 2X TaqMan Gene Expression Mastermix (Applied Biosystems, MA, USA), 7μL of RNAse-free water and 1μL of 10X TaqMan gene expression assay for one of the following per reaction: *IGHG1* (Hs00378340_m1, Cat. No. 4331182, FAM-MGB), *IGHA1* (Hs00733892_m1, Cat. No. 4331182, FAM-MGB), *IGHM* (Hs00378435_m1, Cat. No. 4331182, FAM-MGB) and *ACTB* (Hs01060665_g1, Cat. No. 4331182, FAM-MGB) (Applied Biosystems, MA, USA). *ACTB* (β-actin) was used as a housekeeper gene. Samples were run in triplicate for the genes of interest and housekeeper gene, with appropriate positive and negative (non-template) controls on a Bio-Rad iQ5 qPCR instrument (Bio-Rad, California, USA). Thermal cycler conditions were as follows: hold 50^{0}C for 2 minutes, hold 95^{0}C for 10 minutes followed by 70 X cycles (95^{0}C for 15 second, 60^{0}C for 1 minute), standard ramp rate. Quality control was maintained by assessment of a minimum C_T threshold for *ACTB* for each sample, samples achieving threshold at a C_T of $\geq$45 with poor traces were considered non-specific. Given that the RNA used was derived from DBS and was therefore of a lower concentration and quality compared with RNA extracted from other sources (such as fresh whole blood or purified PBMCs), later than usual amplification was noted for *ACTB* and other genes assessed.

3.9 FUT Polymorphism in silico Analysis

Specificity of the primers used in different publications for genotyping the SNPs rs601338 and rs602662 in *FUT2* and rs778986, rs28362459, rs3894326 and rs3745635 in *FUT3* was assessed by checking the annealing region for each pair of primers using the Ensembl BLAST tool (http://www.ensembl.org/index.html).

3.10 Nested PCR of *FUT2* and *FUT3* Genes

Genomic DNA (extracted from DBS as detailed above) was pre-amplified by PCR using primers specific for *FUT2* and *FUT3* regions containing the SNPs of interest. Thirty nanograms of genomic DNA, 10μL of 1x goTaq colourless buffer (Promega, Madison, WI, USA), 3uL of 10 mM deoxy-ribonucleotide triphosphate (dNTP) (Invitrogen, Carlsbad, CA), 3μg of 1.5mM/μL MgCl2 (Promega, Madison, WI, USA), 2μL of each forward and reverse amplification primer at 10nM (Eurofins), 0.25μL of goTaq DNA polymerase (Promega, Madison, WI, USA) and 26.75μL of distilled water were combined to give a total reaction volume of 50μL. Thermal cycler conditions were as follows: initial denaturation at 95°C for 2 minutes, followed by 30 cycles of: denaturalization 95°C for 30 seconds, annealing 68°C (*FUT2*) or 60°C (*FUT3*) for 30

seconds, and extension 72°C for 1 minute, then final extension at 72°C for 10 minutes. PCR products were visualised in 1% agarose gel.

3.11 *FUT2* and *FUT3* Genotyping using TaqMan Chemistry

TaqMan chemistry (Life Technologies, Carlsbad, CA, USA) was used to genotype two SNPs in *FUT2* (rs601338 (C_2405292_10), rs602662 (C_2405293_10) and two in *FUT3*, rs3894326 (C_801690_10), rs778986 (C_11458475_20). Non-template negative controls, and samples confirmed to have mutant alleles (by Sanger sequencing) were used as positive controls in the assays. Four microliters of amplified PCR product diluted 1:200 were used in the TaqMan reaction according to manufacturer conditions in a final volume of 20µl, (Life Technologies, Carlsbad, CA, USA) and analysed using a Real-Time PCR system under conditions recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA).

3.12 *FUT2* and *FUT3* Genotyping using Sanger Sequencing

The *FUT3* SNPs rs3745635 and rs28362459 were genotyped using 4µl of amplified PCR product diluted 1:80 as a template for Sanger sequencing, which was performed by Eurofins, Munich, Germany. Results were analysed using Mutation Taster software.

3.13 Meta-Analysis and Systematic Review

A systematic review and meta-analysis was conducted using PubMed (http://www.ncbi.nlm.nih.gov/pubmed), Medline Ovid (http://www.ovid.com) and Cochrane Library (http://www.cochranelibrary.com) databases applying the MeSH Terms '*FUT2*', '*FUT3*', 'single nucleotide polymorphism', 'secretor', 'non-secretor', 'Lewis b', 'Lewis b negative'. Minor genotype frequencies for each SNP in published studies and the 1000 Genomes Project (http://www.internationalgenome.org) for selected populations were reviewed. Review Manager 5.0 (2008 Cochrane Collaboration, Oxford, United Kingdom) was used to carry out the statistical analysis.

3.14 Statistical Analyses

Statistical analyses in included studies were performed using SPSS Statistics Version 23 (IBM, USA), GraphPad Prism Version 6.0 (GraphPad Software, San Diego, CA) and Review Manager 5.0 (2008 Cochrane Collaboration, Oxford, United Kingdom).

CHAPTER 4: NEWBORN SCREENING FOR SEVERE FORMS OF PRIMARY IMMUNODEFICIENCY DISEASE MANIFESTED BY T AND/OR B CELL LYMPHOPAENIA

4.1 Introduction and Contextual Statement

The ideal strategy for screening newborns for severe forms of PID has not yet been determined. Most centres are currently screening infants for SCID using a TREC-only assay. However, there are several merits to adopting a screening approach with enables simultaneous detection of both TREC and KREC levels.

This chapter examines the efficacy of a qPCR-based, multiplexed TREC/KREC screening approach in a prospective screening study of almost 60,000 newborns. It is the largest study of its kind to date.

4.2 Publication: Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden – a Two-Year Pilot TREC & KREC Screening Study

This chapter presents the second publication included in this thesis. The following paper, entitled 'Newborn screening for severe primary immunodeficiency diseases in Sweden– a two-year pilot TREC & KREC screening study', by Michela Barbaro, Annika Ohlsson, Stephan Borte, Susanne Jonsson, Rolf Zetterström, Jovanka King, Jacek Winiarski, Ulrika von Döbeln and Lennart Hammarström was published in the peer reviewed journal, Journal of Clinical Immunology, in November 2016 (37(1), 51–60, doi.org/10.1007/s10875-016-0347-5). Supplementary material follows the paper.

ARTICLE METRICS (AS AT 11 JANUARY 2019):

Journal Impact Factor: 3.382

Citations: 34

Altmetrics score: 3

Statement of Authorship

Title of Paper	Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden - A 2-Year Pilot TREC and KREC Screening Study.
Publication Status	Published
Publication Details	Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden-a 2-Year Pilot TREC and KREC Screening Study. Barbaro M, Ohlsson A, Borte S, Jonsson S, Zetterström RH, King J, Winiarski J, von Döbeln U, Hammarström L. J Clin Immunol. 2017 Jan;37(1):51-60. doi: 10.1007/s10875-016-0347-5. Epub 2016 Nov 21. PMID: 27873105

Principal Author

Name of Principal Author (Candidate)	Dr Jovanka King		
Contribution to the Paper	Establishment of databases, data analysis, statistical analyses, manuscript design, writing of manuscript, production of figures and tables, literature review. Total of 1000 research hours contributed to the development & production of this work.		
Overall percentage (%)	25%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.		
Signature		Date	11/4/18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Professor Lennart Hammarström		
Contribution to the Paper	Initiated study, established study design, writing & critical review of manuscript.		
Signature		Date	2018-04-11

Title of Paper	Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden - A 2-Year Pilot TREC and KREC Screening Study.
Publication Status	Published
Publication Details	Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden-a 2-Year Pilot TREC and KREC Screening Study. Barbaro M, Ohlsson A, Borte S, Jonsson S, Zetterström RH, King J, Winiarski J, von Döbeln U, Hammarström L. J Clin Immunol. 2017 Jan;37(1):51-60. doi: 10.1007/s10875-016-0347-5. Epub 2016 Nov 21. PMID: 27873105

Name of Co-Author	Michela Barbaro, M.Sc., PhD
Contribution to the Paper	Implemented the assay used in this study at the Centre for Inherited Metabolic Diseases. Coordinated the daily neonatal screening, collected and analysed the results. Revised the manuscript.
Signature	Date 2018-04-13

Title of Paper	Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden - A 2-Year Pilot TREC and KREC Screening Study.
Publication Status	Published
Publication Details	Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden-a 2-Year Pilot TREC and KREC Screening Study. Barbaro M, Ohlsson A, Borte S, Jonsson S, Zetterström RH, King J, Winiarski J, von Döbeln U, Hammarström L. J Clin Immunol. 2017 Jan;37(1):51-60. doi: 10.1007/s10875-016-0347-5. Epub 2016 Nov 21. PMID: 27873105

Name of Co-Author	Annika Ohlsson, M.Sc, PhD		
Contribution to the Paper	Implemented the assay used in this study at the Centre for Inherited Metabolic Diseases. Coordinated the daily neonatal screening, collected and analysed the results. Revised the manuscript.		
Signature		Date	2018-04-13

Title of Paper	Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden - A 2-Year Pilot TREC and KREC Screening Study.
Publication Status	Published
Publication Details	Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden-a 2-Year Pilot TREC and KREC Screening Study. Barbaro M, Ohlsson A, Borte S, Jonsson S, Zetterström RH, King J, Winiarski J, von Döbeln U, Hammarström L. J Clin Immunol. 2017 Jan;37(1):51-60. doi: 10.1007/s10875-016-0347-5. Epub 2016 Nov 21. PMID: 27873105

Name of Co-Author	Dr Stephan Borte		
Contribution to the Paper	Developed the assay used in this study and was engaged in trouble shooting of the assay during the trial and headed the steering meetings during the study.		
Signature		Date	2018-06-29

Title of Paper	Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden - A 2-Year Pilot TREC and KREC Screening Study.
Publication Status	Published
Publication Details	Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden-a 2-Year Pilot TREC and KREC Screening Study. Barbaro M, Ohlsson A, Borte S, Jonsson S, Zetterström RH, King J, Winiarski J, von Döbeln U, Hammarström L. J Clin Immunol. 2017 Jan;37(1):51-60. doi: 10.1007/s10875-016-0347-5. Epub 2016 Nov 21. PMID: 27873105

Name of Co-Author	Susanne Jonsson, Biomedical Scientist		
Contribution to the Paper	Performed the screening tests at the Centre for Inherited Metabolic Diseases.		
Signature		Date	2018-04-13

Title of Paper	Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden - A 2-Year Pilot TREC and KREC Screening Study.
Publication Status	Published
Publication Details	Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden-a 2-Year Pilot TREC and KREC Screening Study. Barbaro M, Ohlsson A, Borte S, Jonsson S, Zetterström RH, King J, Winiarski J, von Döbeln U, Hammarström L. J Clin Immunol. 2017 Jan;37(1):51-60. doi: 10.1007/s10875-016-0347-5. Epub 2016 Nov 21. PMID: 27873105

Name of Co-Author	Dr Rolf Zetterström		
Contribution to the Paper	Supervised and taken part in the screening at the Centre for Inherited Metabolic Diseases. Reviewed the manuscript.		
Signature		Date	18 04 13

Title of Paper	Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden - A 2-Year Pilot TREC and KREC Screening Study.
Publication Status	Published
Publication Details	Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden-a 2-Year Pilot TREC and KREC Screening Study. Barbaro M, Ohlsson A, Borte S, Jonsson S, Zetterström RH, King J, Winiarski J, von Döbeln U, Hammarström L. J Clin Immunol. 2017 Jan;37(1):51-60. doi: 10.1007/s10875-016-0347-5. Epub 2016 Nov 21. PMID: 27873105

Name of Co-Author	Dr Jacek Winiarski		
Contribution to the Paper	Provided specialist paediatric immunology clinical evaluation and follow-up for all children with a suspected primary immunodeficiency disease. Participated in planning of the study and writing and review of the manuscript		
Signature		Date	April 10, 2018

Title of Paper	Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden - A 2-Year Pilot TREC and KREC Screening Study.
Publication Status	Published
Publication Details	Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden-a 2-Year Pilot TREC and KREC Screening Study. Barbaro M, Ohlsson A, Borte S, Jonsson S, Zetterström RH, King J, Winiarski J, von Döbeln U, Hammarström L. J Clin Immunol. 2017 Jan;37(1):51-60. doi: 10.1007/s10875-016-0347-5. Epub 2016 Nov 21. PMID: 27873105

Name of Co-Author	Dr Ulrika von Döbeln		
Contribution to the Paper	Planned the project together with Lennart Hammarström, wrote the ethical application, took part in and supervised the screening process, revised the manuscript.		
Signature		Date	2018-04-12

J Clin Immunol
DOI 10.1007/s10875-016-0347-5

ORIGINAL ARTICLE

Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden—a 2-Year Pilot TREC and KREC Screening Study

Michela Barbaro [1,2] · Annika Ohlsson [1,3] · Stephan Borte [4,5] · Susanne Jonsson [1] · Rolf H. Zetterström [1,2] · Jovanka King [4,6,7] · Jacek Winiarski [8,9] · Ulrika von Döbeln [1,3] · Lennart Hammarström [4]

Received: 11 July 2016 / Accepted: 18 October 2016

Abstract Newborn screening for severe primary immunodeficiencies (PID), characterized by T and/or B cell lymphopenia, was carried out in a pilot program in the Stockholm County, Sweden, over a 2-year period, encompassing 58,834 children. T cell receptor excision circles (TREC) and kappa-deleting recombination excision circles (KREC) were measured simultaneously using a quantitative PCR-based method on DNA extracted from dried blood spots (DBS), with beta-actin serving as a quality control for DNA quantity. Diagnostic cutoff levels enabling identification of newborns with milder and reversible T and/or B cell lymphopenia were also evaluated. Sixty-four children were recalled for follow-up due to low TREC and/or KREC levels, and three patients with immunodeficiency (Artemis-SCID, ATM, and an as yet unclassified T cell lymphopenia/hypogammaglobulinemia) were identified. Of the positive samples, 24 were associated with prematurity. Thirteen children born to mothers treated with immunosuppressive agents during pregnancy (azathioprine ($n = 9$), mercaptopurine ($n = 1$), azathioprine and tacrolimus ($n = 3$)) showed low KREC levels at birth, which spontaneously normalized. Twenty-nine newborns had no apparent cause identified for their abnormal results, but normalized with time. Children with trisomy 21 ($n = 43$) showed a lower median number of both TREC (104 vs. 174 copies/μL blood) and KREC (45 vs. 100 copies/3.2 mm blood spot), but only one, born prematurely, fell below the cutoff level. Two children diagnosed with DiGeorge syndrome were found to have low TREC levels, but these were still above the cutoff level.

Michela Barbaro, Annika Ohlsson and Stephan Borte contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s10875-016-0347-5) contains supplementary material, which is available to authorized users.

✉ Ulrika von Döbeln
ulrika.vondebeln@karolinska.se

✉ Lennart Hammarström
lennart.hammarstrom@ki.se

[1] Centre for Inherited Metabolic Diseases, Karolinska University Hospital Solna, SE-17176 Stockholm, Sweden

[2] Department of Molecular Medicine and Surgery, Karolinska Institutet, SE-17176 Stockholm, Sweden

[3] Department of Medical Biochemistry and Biophysics, Division of Molecular Metabolism, Karolinska Institutet, SE-17177 Stockholm, Sweden

[4] Department of Clinical Immunology, Karolinska University Hospital Huddinge, SE-14186 Stockholm, Sweden

[5] ImmunoDeficiencyCenter Leipzig (IDCL) at Hospital St. Georg Leipzig, Delitzscher Strasse 141, 04129 Leipzig, Germany

[6] Department of Immunopathology, SA Pathology, Women's and Children's Hospital Campus, North Adelaide, South Australia 5006, Australia

[7] Robinson Research Institute and Discipline of Paediatrics, School of Medicine, University of Adelaide, North Adelaide, South Australia 5006, Australia

[8] Department of Clinical Technology and Intervention, Karolinska Institutet, SE-14186 Stockholm, Sweden

[9] Department of Pediatrics, Karolinska University Hospital Huddinge, SE-14186 Stockholm, Sweden

Published online: 21 November 2016

This is the first large-scale screening study with a simultaneous detection of both TREC and KREC, allowing identification of newborns with both T and B cell defects.

Keywords Newborn screening · primary immunodeficiency diseases · severe combined immunodeficiency · TREC · KREC

Introduction

The purpose of neonatal screening is the early recognition of treatable, mostly genetically determined diseases that manifest with a high rate of morbidity and mortality. Mass screening of newborn infants with dried blood spots (DBS) started in the early 1960s based on a method developed by Guthrie and Susi for diagnosing phenylketonuria [1]. Peripheral blood from a heel stick was blotted onto filter paper (referred to as "Guthrie card") at 3 to 5 days after birth, dried, and sent by mail to centralized laboratories for analysis.

The US congress decided in 2006 to include 29 core and 25 secondary disorders in its newborn screening program based on a priority list of disorders. In recent years, a number of additional disorders, among them severe combined immunodeficiency (SCID), have been included in this list, based on the well-recognized Wilson and Jungner criteria [2]. In Sweden, neonatal screening with DBS was initiated in 1965 and today, a total of 24 disorders are included in the program.

Genetically determined disorders of immunity are commonly referred to as primary immunodeficiencies (PID) and were first recognized clinically over 60 years ago with the identification of X-linked agammaglobulinemia (XLA) [3]. Today, the group of PID includes more than 250 distinct entities, which are divided into T cell deficiencies, B cell deficiencies (the predominant group), combined T and B cell deficiencies, complement defects, and granulocyte defects [4].

Primary immunodeficiencies as a group are not rare diseases, but should be considered in all patients presenting with severe, atypical, or recurrent infections. The overall prevalence is unknown, but varies greatly (from 1:600 for IgA deficiency, 1:20,000 for common variable immunodeficiency, 1:50,000 for SCID, and 1:100,000 for XLA) [4] and references therein [5]. The estimated incidence of severe forms of PID, which require immediate attention, is variable in different populations, but would be expected to be in the order of 5–10 per 100,000 live births.

In 2005, Chan and Puck published a seminal paper, describing the T-cell receptor excision circle (TREC) assay as a tool for large-scale screening for SCID and other T cell lymphopenias [6]. T cell receptor genes are normally edited during T cell differentiation, and the deleted fragments are circularized and do not undergo further replication in dividing cells. Thus, TRECs are a marker of recently formed T cells. The TREC copy number can be determined using a quantitative PCR-based method using DNA extracted from routinely collected DBS. The TREC assay was implemented in the state of Wisconsin in 2008 [7] and its inclusion in the newborn screening programs in the USA was recommended in 2010. Currently, 38 states are screening for SCID using the TREC assay, with the remaining 12 states due to commence in 2017 (Jeffrey Modell Foundation, http://www.info4pi.org/). More than three million newborns have been tested to date [8]. The results show a frequency of SCID of 1/58,000 children (overall 1/7300 with clinically relevant forms of T cell lymphopenia) and a high survival rate (92 %) after treatment.

Congenital B cell lymphopenia can be identified by screening for kappa-deleting recombination excision circles (KREC), the circular by-product of B cell immunoglobulin kappa gene rearrangement [9]. Patients with severe forms of B cell deficiency such as XLA are easily identified by the KREC assay [10] and are treated with regular gammaglobulin infusions, thereby allowing a near to normal life. Furthermore, it has been shown that delayed-onset forms of SCID and other severe forms of PID that present with normal T cell numbers and TREC levels above cutoff in newborn screening programs may be identified using a combined TREC and KREC screening approach [11].

We thus initiated a screening program for all newborns in the county of Stockholm in Sweden using a combined TREC and KREC assay [12]. We previously analyzed 10,058 samples as part of a feasibility study, and one XLA patient was identified (Bruton tyrosine kinase gene mutation, c.1480C>T, p.Gln494Ter), thus demonstrating proof of principle of the combined screening approach [12]. Although a small cohort of Spanish newborns have been screened using the combined TREC/KREC assay [13], this represents the first large-scale prospective study where both T and B cell defects (lymphopenia) can be identified and the results of the first 2 years of the screening program are reported here.

Materials and Methods

Sampling

We carried out a study encompassing samples from all children born in the Stockholm County from November 15, 2013 to November 15, 2015, searching for children with T and B cell lymphopenia, in order to identify those with severe forms of PID. A total of 58,834 samples were analyzed. For 24 additional infants, the parents chose not to participate in the study. The blood sample was collected onto Whatman 903 filter paper as soon as possible after 48 h of age, presently at a mean age of 2.8 days, and was then mailed to the laboratory. The average age at recall for a positive screening result was 6 days.

The regional ethical board in Stockholm approved the study (Ethical permit 2013/414-31/4).

TREC/KREC Screening Assay

The TREC/KREC newborn screening assay described previously [12] was utilized, with minor technical modifications. Briefly, DNA was extracted from single 3.2-mm punches of the original DBS in a 96-well format, and quantitative triplex real-time qPCR for TREC, KREC, and beta-actin (ACTB) was performed using a ViiA7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) on DNA eluates as previously described [12]. The qPCR procedure was optimized using custom reagents provided by Affymetrix (Santa Clara, CA, USA). TREC and KREC levels were determined per 3.2-mm punch. Amplification of ACTB was used to assess that a sufficient quantity of DNA was extracted from each DBS, but this was disregarded if TREC and KREC copy numbers were above the cutoff level. The samples were initially analyzed once, and if the value was below the cutoff, they were reanalyzed in duplicate. Samples in which TREC and/or KREC levels were below cutoff in association with a reduction in ACTB copy number were considered inconclusive. Cutoff levels for TREC/KREC were adjusted during the study based on the yield of positive samples.

Genetic Characterization of Patients with Low TREC/KREC Levels

For the three children with suspected PID, whole exome sequencing (WES) was performed, followed by confirmatory Sanger sequencing. DNA purification, library preparation, read mapping, variant determination, and analysis of WES data were performed as described previously [14, 15]. Sequences were generated as 90-bp pair-end reads and aligned to the human genome reference (UCSC hg 19 version, build 37.1) by using the SOAP aligner (soap 2.21) software. Duplicated reads were filtered out and only uniquely mapped reads were kept for subsequent analysis. The SOAPsnp (version 1.03) software was subsequently used with default parameters to assemble the consensus sequence and call genotypes in target regions. For single nucleotide polymorphism (SNP) quality control, low-quality SNPs that met one of the four following criteria were filtered out: (1) a genotype quality of less than 20, (2) a sequencing depth of less than 4, (3) an estimated copy number of more than 2, and (4) a distance from the adjacent SNPs of less than 5 bp. Small insertions/deletions were detected by using the Unified Genotype tool from GATK (version v1.0.4705) after alignment of quality reads to the human reference genome using BWA (version 0.5.9-r16).

Statistical Analyses

Statistical analyses were performed using GraphPad Prism Version 6.0 (GraphPad Software, San Diego, CA). Given the nonparametric distribution of the data, the Mann-Whitney *U* test was used for group comparison analyses, and the Spearman correlation coefficient was calculated for correlation analyses. Differences were considered statistically significant when the p value was less than 0.05.

Results

Screening Overview

In total, 58,834 newborns were screened. Samples with TREC and/or KREC copies below the cutoff values were considered "abnormal" (positive), whereas those that also had reduced ACTB copy numbers were considered to be "inconclusive." Initially, 572 samples were found to be either abnormal (positive) or inconclusive ($n = 399$ and $n = 173$, respectively) (Fig. 1). After repeat testing on the original DBS, 64 abnormal (positive) samples remained, i.e., 99.9 % were considered normal and were not evaluated further. A total of 13 patients were recalled for repeat sampling due to poor quality of their initial DBS sample.

The definition of "low" levels of TREC and KREC copies will determine how many samples are considered positive. The dilemma is to avoid unnecessary recalls while still capturing all patients with a severe form of PID (i.e., SCID and XLA), as well as additional patients with profound disturbance of T and B cell homeostasis at birth. Three different cutoff levels for recall were used during the study as the limits were adjusted based on the number and outcome of the recalls. During the first period, a cutoff level of 15 copies/3.2 mm blood spot or below for TREC and 10 copies/3.2 mm blood spot blood or below for KREC was applied. In total, 16,582 children were screened and 35 were considered to have a positive result. However, the vast majority recovered spontaneously (Supplementary Table 1) and the cutoff was subsequently lowered to a TREC level of 8 copies/3.2 mm blood spot or below and a KREC level of 4 copies/3.2 mm blood spot blood or below. Another 28,298 children were screened during this period and 11 were found to be positive, among them PID patients 1 and 2. As these cutoffs were ultimately considered too stringent, they were finally changed to a TREC level of 10 copies/3.2 mm blood spot or below and a KREC level of 6 copies/3.2 mm blood spot or below. Altogether, 13,954 children were subsequently screened and 18 were found to be positive, among them PID patient 3.

The patients with TREC/KREC levels below the cutoff (Supplementary Table 1) were referred to a pediatrician specialized in the diagnosis and management of PID. The patient with absent TREC and KREC was immediately hospitalized whereas in the vast majority of patients, a follow-up sample for TREC and KREC analysis was collected within 2–10 weeks (Table 1) depending on the clinical urgency and gestational age. The recall was almost exclusively due to

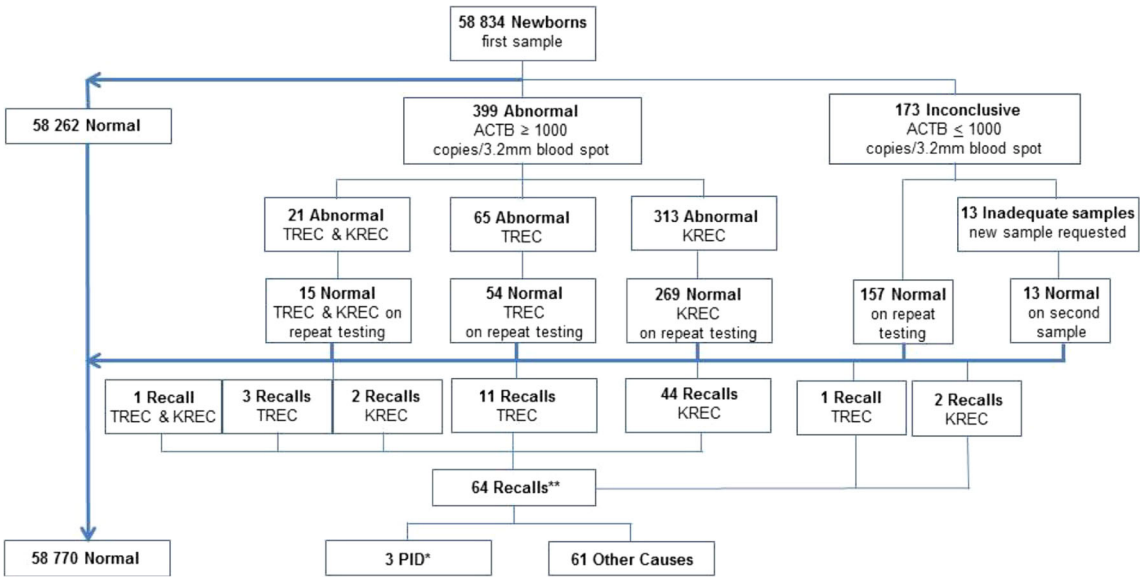


Fig. 1 Summary of all newborn screening results between 15 November 2013 and 15 November 2015. *Two infants had low TREC levels, and one had low TREC and KREC levels. **See Fig. 2

low KREC levels, and these patients were usually seen and resampled at around 3 weeks of age. In the majority of cases, the TREC/KREC values had normalized at the time of resampling (Supplementary Table 1) and only three patients (approximately 1/20,000) were referred for flow cytometric (fluorescence-activated cell sorting, FACS) analysis and genetic work-up (WES). In the majority of recalled patients, follow-up investigation with FACS analysis was not performed, as it was not deemed clinically relevant. To date, none of the infants in whom KREC/TREC levels normalized have subsequently developed features of PID.

Abnormal (Positive) TREC/KREC Results

Altogether, 64 children were recalled for follow-up due to low TREC and/or KREC levels and three patients with severe primary immunodeficiency were identified (Supplementary Table 1). Among the positive samples (Fig. 2), several infants had more than one potential etiological factor contributing to their low TREC and/or KREC levels. A total of 24 infants were premature, including one with trisomy 21 and 11 were a twin or triplet. Furthermore, 13 infants were born to mothers receiving immunosuppressive therapy. Twenty-nine had no apparent cause identified and the TREC/KREC levels in the children tested ($n = 27$) normalized with time. Resampling was declined for four of the 64 children recalled.

The 13 children born to mothers who were treated with immunosuppressive agents during pregnancy (azathioprine ($n = 9$), mercaptopurine ($n = 1$), azathioprine and tacrolimus ($n = 3$)) showed low KREC levels at birth, which however spontaneously normalized after 2–10 weeks. Two children from a mother treated with azathioprine, born almost 2 years apart, both showed low KREC at birth.

Other Conditions Influencing the Screening Result

Children with trisomy 21 ($n = 43$) showed a lower median number of both TREC (104 vs. 174 in the total material) and KREC (45 vs. 100), but only one child, born prematurely, fell below the cutoff levels applied at the time of testing. Two children were diagnosed with DiGeorge syndrome in the Department of Clinical Genetics, and both had a low TREC level but these were still above the cutoff level at the time of testing.

Among the 3457 infants born prematurely (prior to 37 weeks gestation), 24 screened positive with low TREC ($n = 12$), KREC ($n = 11$) levels, or both ($n = 1$) (Supplementary Table 1). Although T cells appear to mature late in fetal life, TREC and KREC levels were surprisingly "normal" in the majority of children, even at very low gestational ages (Table 2), albeit as a group they were still significantly below the levels in children born at term ($p < 0.0001$, Mann-Whitney U test) (Fig. 3).

A total of 896 twin pairs and 16 triplet sets ($n = 1840$) were identified in the cohort, and the TREC and/or KREC values differed markedly between the newborns in both those siblings with low levels (Table 3) and in those with values within the normal range (Fig. 4).

To the best of our knowledge, no patients with severe PID characterized by absent T or B cells at birth have been missed to date among those infants included in the screening program.

PID Patients

Three patients were found to have repeatedly low levels of both TREC and KREC ($n = 1$) or TREC alone ($n = 2$) (Table 1) and

Table 1 Serial TREC and KREC levels in PID patients with abnormal screening test results

				TREC copies/3.2 mm blood spot				KREC copies/3.2 mm blood spot			
Case	Sex	Gestational age (weeks)	Diagnosis	Result 1	Result 2	Result 3	Result 4	Result 1	Result 2	Result 3	Result 4
1	Male	34	Artemis deficiency	Day 2 0	Day 8 0	Day 14 0	Day 24 0	Day 2 0	Day 8 0	Day 14 0	Day 24 0
2	Male	39	Ataxia-telangiectasia	Day 2 5	Day 25 5	Day 43 3	Day 51 3	Day 2 7	Day 25 10	Day 43 21	Day 51 19
3	Male	36	Unknown genetic defect[a]	Day 2 7	Day 27 4	Day 34 5	Day 94 1	Day 2 205	Day 27 732	Day 34 504	Day 94 250

[a] T cell lymphopenia and hypogammaglobulinemia. At follow-up at 15 months of age, the child's hypogammaglobulinemia had resolved but a persistent idiopathic CD3+ T cell lymphopenia (absolute CD3+ count = 0.9×10^9 /L) was evident

were considered to have a severe immunodeficiency disorder. The first patient had a homozygous splice-site mutation in Artemis (*DLCLRE1C* c.333+2T>G) with absent protein expression and underwent a successful stem cell transplantation at the age of 2 months. The second child shows clinical features of ataxia-telangiectasia and carries compound heterozygous mutations in *ATM*, where the mother contributed a c.3673C>T mutation resulting in a stop codon (p.Gln1225Ter) and the father contributed a c.8653_8654insT mutation resulting in a Val2886CysfsTer10 mutation. The third child was found to have T cell lymphopenia and hypogammaglobulinemia, but genetic analysis (including whole genome sequencing) has not as yet revealed any causative mutation in known PID genes. The results of immunological investigations for the three PID patients identified are given in Supplementary Table 2.

Discussion

Since 2008, TREC analysis has been the method of choice for screening of newborns for severe forms of primary T cell lymphopenia [6, 7, 16], a method that will capture most, but not all, children with SCID (exceptions being patients with mutations in *ZAP70* [17, 18], *MHC* class II [19–21], and *ADA* (delayed-onset disease [11]). We subsequently developed a triplex method that also includes KREC analysis to assess for potential B cell lymphopenia in DBS samples. This method can successfully identify patients with XLA [12], selected patients with delayed-onset adenosine deaminase deficiency (ADA) [11], Nijmegen-breakage syndrome (NBS) [12], and purine nucleoside phosphorylase (PNP) deficiency [22]. The combined assay has recently also been used to screen patients with SCID [23, 24], ATM [12, 25], Wiskott-Aldrich syndrome (WAS) [26], DiGeorge syndrome [27–29], and trisomy 21 [30], and it has been suggested to be included in routine screening of newborns for primary immunodeficiency [31–34]. KREC levels have also been used to monitor immune reconstitution after bone marrow/stem cell transplantation [35–37].

Ours is the largest study to date using a combined TREC/KREC assay for newborn screening. One SCID patient was identified (and successfully transplanted) in our cohort of 58,000 infants, comparable with the data published by Kwan et al. on a very large US cohort [8]. One patient with ATM was also identified. This disorder is very rare, with an estimated incidence of 1 in 200,000 according to recent figures from the

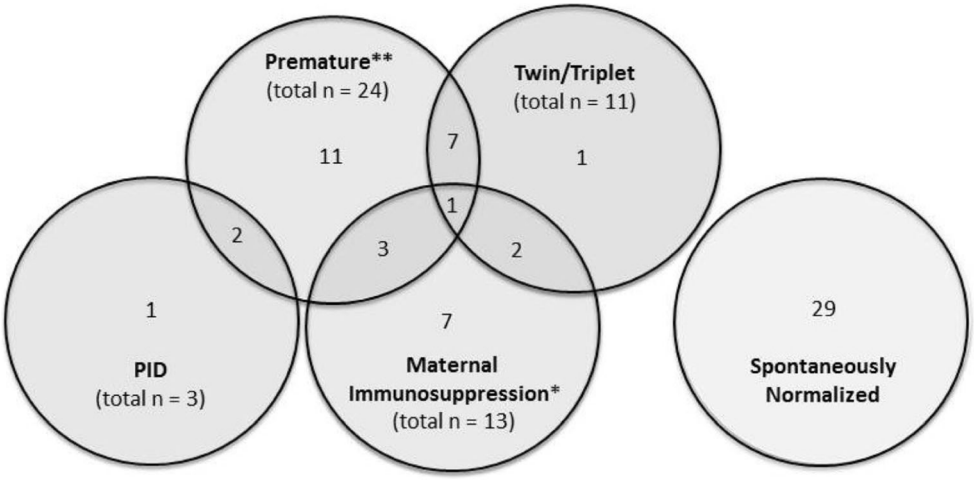


Fig. 2 Characteristics of the 64 infants with abnormal screening results recalled for repeat testing. *Azathioprine ($n = 9$), mercaptopurine ($n = 1$), azathioprine + tacrolimus ($n = 3$). **One infant who was premature also had trisomy 21

Table 2 Mean TREC and KREC levels at different gestational ages

Gestational age (weeks)	Infants (*n*)	Mean TREC level (copies/3.2 mm blood spot)	Mean KREC level (copies/3.2 mm blood spot)
20	1	262	139
21	2	97	180
22	3	84	97
23	23	53	52
24	31	68	58
25	38	64	70
26	50	71	62
27	58	89	65
28	53	93	76
29	97	114	91
30	97	139	110
31	122	134	99
32	199	146	90
33	283	139	87
34	390	142	93
35	663	153	97
36	1347	156	103
Total	3457		

USA [38]. It will be interesting to determine if this finding is due to serendipity or if the disease is more frequent in Scandinavia. Our third patient showed a T cell lymphopenia of unknown cause. Such cases have been previously described in studies in the USA with incidences varying between 3 and 4 per 100,000 children in different states and with a predicted incidence of 1 in 160,000 in a British study (Professor Bobby Gaspar, Great Ormond Street Hospital, personal communication). Recently, during the third year of screening, yet another SCID patient (ADA deficiency) and a patient with severe T cell lymphopenia have been identified.

The number of referrals for flow cytometric analysis in our cohort was low (approximately 1:20,000), markedly lower than that reported in the US study of Kwan et al. [8] (ranging from 1/735 to 1/7500 in the different states). This is a reflection of the strict resampling strategy that was applied to children with low,

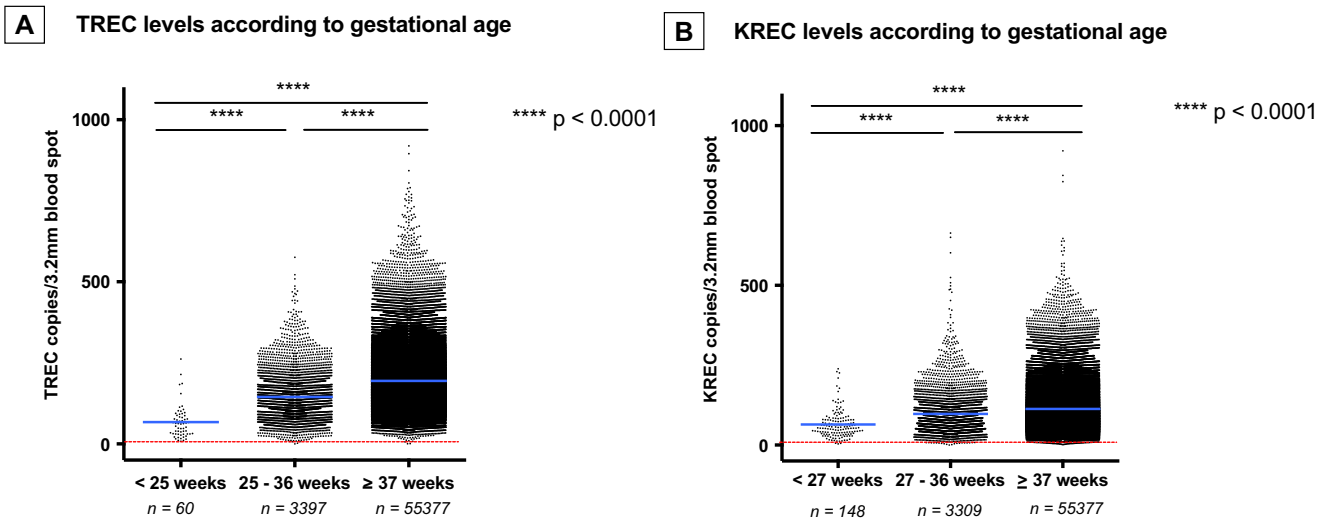


Fig. 3 Comparison of TREC levels (**a**) in infants born prior to 25 weeks, between 25 and 36 weeks, and at term (≥37 weeks) gestation. Comparison of KREC levels (**b**) in infants born prior to 27 weeks, between 27 and 36 weeks, and at term (≥37 weeks) gestation. Each *point* represents one infant and the number of infants in each group is indicated. The *horizontal blue line* indicates the mean value of all samples. The *dashed horizontal red line* represents the cutoff value for TREC (<10 copies/3.2 mm blood spot) and KREC (<6 copies/3.2 mm blood spot) levels, respectively. ****$p < 0.0001$, Mann-Whitney *U* test

Table 3 Discrepant TREC and KREC levels in twin and triplet sets with abnormal screening results

	Gestational age (weeks)	Sex	Twin/ triplet	Age at first sampling (days)	TREC copies/3.2 mm blood spot	KREC copies/3.2 mm blood spot	Comments
1a[a]	38	Female	TW I	3	1	18	Deceased
1b	38	Female	TW II	3	138	161	
2a	34	Male	TW I	2	51	259	
2b[a]	34	Male	TW II	2	13	92	
3a[a]	25	Female	TW I	0	5	25	Deceased
3b	25	Female	TW II	0	19	178	
4a	29	Male	TR I	3	20	42	
4b[a]	29	Female	TR II	3	15	29	
4c	29	Female	TR III	3	70	56	
5a	29	Male	TW I	2	76	130	
5b[a]	29	Male	TW II	2	5	16	
6a	27	n.a	TR I				Deceased
6b	27	n.a	TR II				Deceased
6c[a]	27	Male	TR III	3	5	28	
7a[a]	38	Female	TW I	3	149	6	
7b[a]	38	Female	TW II	3	132	5	
8a	32	Female	TW I	2	77	40	
8b[a]	32	Female	TW II	2	13	5	
9a	24	Male	TW I	2	16	68	
9b[a]	24	Female	TW II	2	9	13	
10a	23	n.a	TW I				Deceased
10b[a]	23	Female	TW II	2	7	11	

TW twin 1, *TR* triplet, *n.a.* not available

[a] Recalled twin/triplet

but not absent TREC or KREC levels. These results also indicate that the addition of KREC testing to the TREC assay in a newborn screening program does not critically drive the demand for second tier testing nor does it increase the fiscal impact of the test itself due to the minimal additional cost of multiplexing.

Prematurity has been considered a predisposing factor for low TREC numbers in studies in American infants [8]. The TREC and KREC levels in our study did show a downward trend with decreasing gestational age, although in the vast majority, the levels were above the cutoff, even in markedly

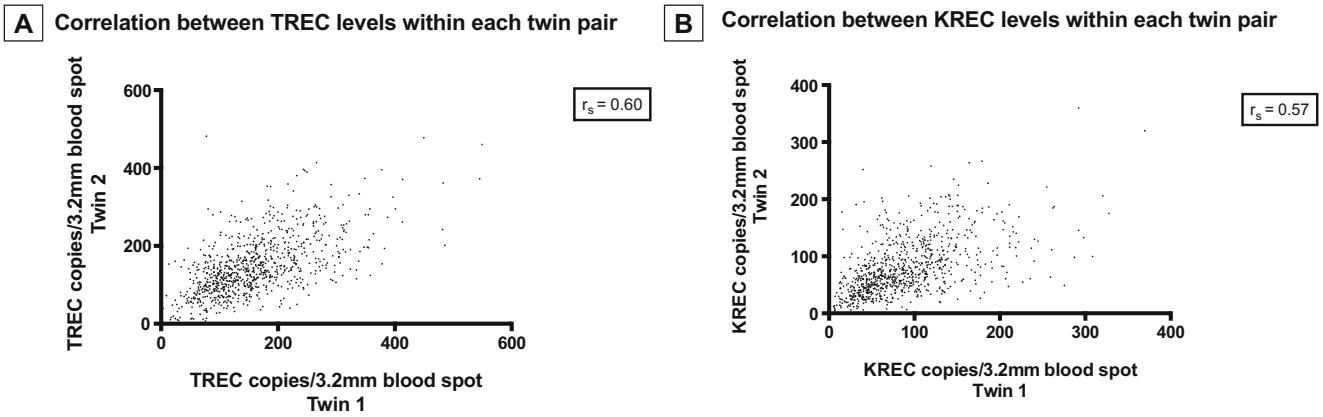


Fig. 4 Correlation between TREC (**a**) and KREC (**b**) levels within all 896 twin pairs. Each *point* represents one twin pair, where the TREC value for twin 1 (*x axis*) is plotted against the TREC value for twin 2 (*y axis*) (**a**). KREC levels for each pair are similarly depicted (**b**). Overall, there was poor correlation between specimen pairs, with a calculated Spearman correlation coefficient (R_s) of 0.6 for TREC and 0.57 for KREC ($p < 0.0001$)

premature infants. This notion was also recently supported [13] in a small Spanish cohort of newborns.

Twins and triplets were markedly overrepresented among the positive samples ($n = 11/64$), potentially reflecting prematurity in the majority of cases ($n = 8/11$). There was a notable difference in TREC and KREC levels within twin pairs returning normal screening results. Although information regarding zygosity was not available, it is expected that one in four pairs is monozygotic and therefore genetically identical, and this observation will thus be further investigated in the future studies.

Thirteen children with low KREC levels were born to 12 mothers who had been treated with azathioprine during pregnancy, three of whom were concomitantly receiving tacrolimus. Two children born to the same azathioprine-treated mother, 2 years apart, both had low KREC levels. Low KREC levels were also recently noted by de Felipe et al. [13] in a small cohort of Spanish children born to mothers treated with azathioprine. As azathioprine may cross the placenta (and is mutagenic), it is generally contraindicated during pregnancy unless used for treatment of severe disease. As B lymphocytes are markedly more sensitive to drug-induced apoptosis than T lymphocytes [39], the selectivity for a reduction in KREC copies alone is not surprising.

We anticipated that some infants with trisomy 21 would screen positive since they may have low B cell and/or T cell production [30]. However, only one of the 43 infants screened during the 2-year period had a KREC level below cutoff, although infants with trisomy 21 had generally lower levels than those in the general newborn population. In Sweden, the estimated frequency of chromosome 22q deletions is around 1/4000, suggesting that some 14–15 children in the screened cohort would suffer from this form of PID. However, less than 10 % of chromosome 22q deletion patients had pronounced T cell lymphopenia (measured as low TREC levels) and an additional 5–10 % had levels close to the cutoff. Thus, we would have expected only one to two children to be identified in our tested cohort, which is not statistically different from the present result (no child identified).

Most of the children in our study were recalled due to low KREC levels. This was expected, as we did not know what the "true" cutoff level for children with XLA would be and thus applied a fairly large safety margin. Ongoing studies in our laboratory, analyzing KREC levels in a large cohort of children with mutation proven XLA, may allow further adjustment of the recall level of KREC, thus reducing the efforts and costs associated with "false" positive results.

Newborn screening for metabolic diseases has undergone major changes during the past decades with inclusion of numerous very rare diseases in the program. We anticipate a similar development in the PID screening field where the KREC may ultimately be added to the TREC assay currently used in several national screening programs. The decision regarding implementation of TREC vs. combined TREC/KREC screening can be guided by considering the advantages and disadvantages of each screening approach. The advantages for adding KREC screening include the identification of children with XLA, late onset ADA, some patients with NBS, and other selected forms of PID whose disease may be undetected by TREC screening alone [8, 23, 40]. In addition, it may assist in distinguishing patients with SCID caused by deficiency in the production of T as well as B cells or only T cells, thus aiding in the diagnostic process. On the negative side, increased costs associated with the additional KREC assay may be considered a disadvantage of the combined screening strategy. With the possibility to multiplex PCR reactions, the individual cost for additional screening markers such as KREC in an existing TREC screening system is negligible (<€0.10 per newborn), but associated costs incurred in follow-up and further testing for infants with abnormal screening results should also be considered. However, it should be noted that the recall rate using the combined TREC/KREC assay is similar to that of TREC screening alone [8], with a lower rate of follow-up FACS studies than in other published studies to date.

As markers for other genetic diseases have been proposed which can be multiplexed with the TREC/KREC system, future efforts may be focused on the extension of existing newborn screening tests for PID [26]. This may potentially include complement deficiencies (using DBS eluates as described by Janzi et al. [41] for C3 deficiencies and Hamsten et al. [42] for C2 deficiencies) and granulocyte defects, thus allowing coverage of a majority of the primary immunodeficiency diseases. Targeted exome sequencing or even whole genome sequencing of newborns may ultimately be considered, which could lead to identification of additional defects of immunity and a large number of other inherited, potentially fatal diseases.

Acknowledgments This work was supported by the Jeffrey Modell Foundation and grants provided by the Stockholm county (ALF project 20140481) and by an unrestricted research grant from the Baxalta company (BT12-000437). We wish to express our gratitude to Katarina Carlsson, Jenny Ljusberg-Sjölander, and Fanny Huynh for their excellent laboratory work.

Authorship Contributions Michela Barbaro, Annika Ohlsson, and Susanne Jonsson performed the screening in the Center for Metabolic Diseases under the supervision of Rolf H. Zetterström and Ulrika von Döbeln.

Jacek Winiarski is a pediatrician specialized in PID and all children with a suspected PID were referred to him for evaluation and follow-up.

Stephan Borte developed the assay used in this study and was engaged in trouble shooting of the assay during the trial and headed the steering meetings during the study.

Jovanka King and Lennart Hammarström wrote the paper.

Compliance with Ethical Standards All procedures performed in studies involving human participants were in accordance with the ethical standards of the regional ethical board in Stockholm (Ethical permit 2013/414-31/4) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Guthrie R, Susi A. A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. Pediatrics. 1963;32:338–43.
2. Wilson J, Jungner G. The principles and practice of screening for disease. Geneva: World Health Organization; 1968.
3. Bruton OC. Agammaglobulinemia. Pediatrics. 1952;9(6):722–8.
4. Al-Herz W, Bousfiha A, Casanova JL, Chatila T, Conley ME, Cunningham-Rundles C, et al. Primary immunodeficiency diseases: an update on the classification from the international union of immunological societies expert committee for primary immunodeficiency. Front Immunol. 2014;5:162.
5. Bousfiha AA, Jeddane L, Ailal F, Benhsaien I, Mahlaoui N, Casanova JL, et al. Primary immunodeficiency diseases worldwide: more common than generally thought. J Clin Immunol. 2013;33(1):1–7.
6. Chan K, Puck JM. Development of population-based newborn screening for severe combined immunodeficiency. J Allergy Clin Immunol. 2005;115(2):391–8.
7. Routes JM, Grossman WJ, Verbsky J, Laessig RH, Hoffman GL, Brokopp CD, et al. Statewide newborn screening for severe T-cell lymphopenia. JAMA. 2009;302(22):2465–70.
8. Kwan A, Abraham RS, Currier R, Brower A, Andruszewski K, Abbott JK, et al. Newborn screening for severe combined immunodeficiency in 11 screening programs in the United States. JAMA. 2014;312(7):729–38.
9. Nakagawa N, Imai K, Kanegane H, Sato H, Yamada M, Kondoh K, et al. Quantification of kappa-deleting recombination excision circles in Guthrie cards for the identification of early B-cell maturation defects. J Allergy Clin Immunol. 2011;128(1):223–5. e2.
10. Borte S, Wang N, Oskarsdóttir S, von Döbeln U, Hammarström L. Newborn screening for primary immunodeficiencies: beyond SCID and XLA. Ann N Y Acad Sci. 2011;1246:118–30.
11. Speckmann C, Neumann C, Borte S, la Marca G, Sass JO, Wiech E, et al. Delayed-onset adenosine deaminase deficiency: strategies for an early diagnosis. J Allergy Clin Immunol. 2012;130(4):991–4.
12. Borte S, von Döbeln U, Fasth A, Wang N, Janzi M, Winiarski J, et al. Neonatal screening for severe primary immunodeficiency diseases using high-throughput triplex real-time PCR. Blood. 2012;119(11):2552–5.
13. de Felipe B, Olbrich P, Lucenas JM, Delgado-Pecellin C, Pavon-Delgado A, Marquez J, et al. Prospective neonatal screening for severe T- and B-lymphocyte deficiencies in Seville. Pediatr Allergy Immunol. 2016;27(1):70–7.
14. Alkhairy OK, Perez-Becker R, Driessen GJ, Abolhassani H, van Montfrans J, Borte S, et al. Novel mutations in TNFRSF7/CD27: clinical, immunologic, and genetic characterization of human CD27 deficiency. J Allergy Clin Immunol. 2015;136(3):703–12.e10.
15. Fang M, Abolhassani H, Lim CK, Zhang J, Hammarström L. Next generation sequencing data analysis in primary immunodeficiency disorders—future directions. J Clin Immunol. 2016.
16. Puck JM, Group SNSW. Population-based newborn screening for severe combined immunodeficiency: steps toward implementation. J Allergy Clin Immunol. 2007;120(4):760–8.
17. Grazioli S, Bennett M, Hildebrand KJ, Vallance H, Turvey SE, Junker AK. Limitation of TREC-based newborn screening for ZAP70 severe combined immunodeficiency. Clin Immunol. 2014;153(1):209–10.
18. Hauck F, Blumenthal B, Fuchs S, Lenoir C, Martin E, Speckmann C, et al. SYK expression endows human ZAP70-deficient CD8 T cells with residual TCR signaling. Clin Immunol. 2015;161(2):103–9.
19. Kuo CY, Chase J, Garcia Lloret M, Stiehm ER, Moore T, Aguilera MJ, et al. Newborn screening for severe combined immunodeficiency does not identify bare lymphocyte syndrome. J Allergy Clin Immunol. 2013;131(6):1693–5.
20. Lev A, Simon AJ, Broides A, Levi J, Garty BZ, Rosenthal E, et al. Thymic function in MHC class II-deficient patients. J Allergy Clin Immunol. 2013;131(3):831–9.
21. Lyttle A, Roifman C, Dadi H, Wright N, Kavadas F. MHC class II deficiency in the Dene native population: a case report highlighting pitfalls in diagnosis and treatment. Allergy, Asthma Clin Immunol. 2014;10(supplement 1):A1.
22. la Marca G, Canessa C, Giocaliere E, Romano F, Malvagia S, Funghini S, et al. Diagnosis of immunodeficiency caused by a purine nucleoside phosphorylase defect by using tandem mass spectrometry on dried blood spots. J Allergy Clin Immunol. 2014;134(1):155–9.
23. Somech R, Lev A, Simon AJ, Korn D, Garty BZ, Amariglio N, et al. Newborn screening for severe T and B cell immunodeficiency in Israel: a pilot study. Isr Med Assoc J. 2013;15(8):404–9.
24. Tamura S, Higuchi K, Tamaki M, Inoue C, Awazawa R, Mitsuki N, et al. Novel compound heterozygous DNA ligase IV mutations in an adolescent with a slowly-progressing radiosensitive-severe combined immunodeficiency. Clin Immunol. 2015;160(2):255–60.
25. Kraus M, Lev A, Simon AJ, Levran I, Nissenkorn A, Levi YB, et al. Disturbed B and T cell homeostasis and neogenesis in patients with ataxia telangiectasia. J Clin Immunol. 2014;34(5):561–72.
26. Borte S, Meeths M, Liebscher I, Krist K, Nordenskjöld M, Hammarström L, et al. Combined newborn screening for familial hemophagocytic lymphohistiocytosis and severe T- and B-cell immunodeficiencies. J Allergy Clin Immunol. 2014;134(1):226–8.
27. Lingman Framme J, Borte S, von Döbeln U, Hammarström L, Oskarsdóttir S. Retrospective analysis of TREC based newborn screening results and clinical phenotypes in infants with the 22q11 deletion syndrome. J Clin Immunol. 2014;34(4):514–9.
28. Froňková E, Klocperk A, Svatoň M, Nováková M, Kotrová M, Kayserová J, et al. The TREC/KREC assay for the diagnosis and monitoring of patients with DiGeorge syndrome. PLoS One. 2014;9(12), e114514.
29. Dar N, Gothelf D, Korn D, Frisch A, Weizman A, Michaelovsky E, et al. Thymic and bone marrow output in individuals with 22q11.2 deletion syndrome. Pediatr Res. 2015;77(4):579–85.
30. Verstegen RH, Borte S, Bok LA, van Zwieten PH, von Döbeln U, Hammarström L, et al. Impact of Down syndrome on the performance of neonatal screening assays for severe primary immunodeficiency diseases. J Allergy Clin Immunol. 2014;133(4):1208–11.
31. Borte S, von Döbeln U, Hammarström L. Guidelines for newborn screening of primary immunodeficiency diseases. Curr Opin Hematol. 2013;20(1):48–54.
32. Chiarini M, Zanotti C, Serana F, Sottini A, Bertoli D, Caimi L, et al. T-cell receptor and K-deleting recombination excision circles in newborn screening of T- and B-cell defects: review of the literature and future challenges. J Public Health Res. 2013;2(1):9–16.
33. Buelow BJ, Routes JM, Verbsky JW. Newborn screening for SCID: where are we now? Expert Rev Clin Immunol. 2014;10(12):1649–57.
34. Gaspar HB, Hammarström L, Mahlaoui N, Borte M, Borte S. The case for mandatory newborn screening for severe combined immunodeficiency (SCID). J Clin Immunol. 2014;34(4):393–7.
35. Mensen A, Ochs C, Stroux A, Wittenbecher F, Szyska M, Imberti L, et al. Utilization of TREC and KREC quantification for the monitoring of early T- and B-cell neogenesis in adult patients after allogeneic hematopoietic stem cell transplantation. J Transl Med. 2013;11:188.

36. Nakatani K, Imai K, Shigeno M, Sato H, Tezuka M, Okawa T, et al. Cord blood transplantation is associated with rapid B-cell neogenesis compared with BM transplantation. Bone Marrow Transplant. 2014;49(9):1155–61.
37. Valotti M, Sottini A, Lanfranchi A, Bolda F, Serana F, Bertoli D, et al. Long-lasting production of new T and B cells and T-cell repertoire diversity in patients with primary immunodeficiency who had undergone stem cell transplantation: a single-centre experience. J Immunol Res. 2014;2014:240453.
38. Mallott J, Kwan A, Church J, Gonzalez-Espinosa D, Lorey F, Tang LF, et al. Newborn screening for SCID identifies patients with ataxia telangiectasia. J Clin Immunol. 2013;33(3):540–9.
39. Dimitriu A, Fauci AS. Activation of human B lymphocytes. XI. Differential effects of azathioprine on B lymphocytes and lymphocyte subpopulations regulating B cell function. J Immunol. 1978;121(6):2335–9.
40. Chien YH, Chiang SC, Chang KL, Yu HH, Lee WI, Tsai LP, et al. Incidence of severe combined immunodeficiency through newborn screening in a Chinese population. J Formos Med Assoc. 2015;114(1):12–6.
41. Janzi M, Sjöberg R, Wan J, Fischler B, von Döbeln U, Isaac L, et al. Screening for C3 deficiency in newborns using microarrays. PLoS One. 2009;4(4), e5321.
42. Hamsten C, Skattum L, Truedsson L, von Döbeln U, Uhlén M, Schwenk JM, et al. Heat differentiated complement factor profiling. J Proteomics. 2015;126:155–62.

4.3 Supplementary Material

Table 4: Characteristics, screening results and further investigation results for 64 newborns recalled for repeat testing

Cutoff used for each time period	Patient	Gestational Age	Sex	Age at 1st sample (days)	Mean 1st sample TREC (run in triplicate)	Mean 1st sample KREC (run in triplicate)	Age at 2nd sample (days)	Mean 2nd sample TREC (run in triplicate)	Mean 2nd sample KREC (run in triplicate)	Diagnosis/Comments
cut off TREC ≤15 KREC ≤10 n= 16 582	1	33	M	2	77	0	9	137	0	Level day 26 TREC 172 and KREC 240
	2	39	M	2	118	8	10	192	23	
	3§	41	F	3	105	3	20	166	52	
	4	38	F (TW I)	3	1	18	n.a.			Placental abruption, asphyxia, syndromic features, deceased
	5	40	M	3	226	2	15	352	1	Level day 58 TREC 241 and KREC 18
	6	40	M	3	97	5	12	189	9	Azathioprine
	7	39	F	3	161	1	15	136	44	Azathioprine
	8	25	F	3	38	3	13	119	33	Premature
	9	37	M	2	112	6	18	252	9	No additional sampling, no presentation with infections as per available records
	10	39	M	4	81	8	19	140	111	
	11#	38	M	2	184	9	n.a.			No presentation with infections as per available records
	12	34	M (TW II)	2	13	92	12	162	361	Premature
	13	38	F	2	12	45	19	57	205	
	14	39	M	3	174	6	17	525	263	
	15	41	M	2	117	6	16	229	98	
	16	41	M	2	79	3	22	191	144	Mercaptopurine
	17	37	M	2	78	2	23	177	156	
	18	38	F	2	77	9	47	127	274	
	19	40	F	2	78	9	32	130	262	
	20	39	F	2	24	8	38	73	377	Azathioprine
	21	42	F	2	142	5	25	173	110	
	22	36	M	2	43	2	36	84	473	Premature, Azathioprine, Tacrolimus
	23	25	F (TW I)	0	5	23	n.a.			Premature, deceased
	24	39	F	2	141	6	43	251	570	Azathioprine
	25	29	F (TR II)	3	15	29	38	134	352	Premature, Azathioprine
	26	39	M	4	234	1	32	410	230	
	27	41	M	2	212	10	24	254	79	
	28	42	M	2	156	9	39	270	197	
	29	41	F	2	248	6	37	379	653	
	30	42	M	2	162	4	28	339	483	

	31#	35	F	3	67	3	n.a.			Premature, no presentation with infections as per available records
	32	40	F	3	271	6	27	355	484	
	33	32	M	3	13	13	n.a.			Premature. Level day 772 TREC 86 and KREC 246
	34	41	M	2	103	8	23	211	323	
	35	38	F	2	153	9	22	409	214	Azathioprine
cut off TREC ≤8 KREC ≤4 n= 28 298	36	29	M (TW II)	2	5	16	11	51	33	Premature
	37	37	F	2	244	3	24	354	333	
	38	40	M	2	52	3	28	135	172	
	39	34	M	2	0	0	7	0	0	Premature, SCID (Artemis), BMT at 2 months of age, alive and well
	40	27	M (TR III)	3	5	28	28	26	106	Premature
	41	38	F	2	134	4	24	225	380	
	42	39	M	2	5	7	20	5	10	Ataxia-telangiectasia mutation, IgAD, asymptomatic at 1 year of age
	43	40	F	2	150	4	21	306	264	
	44	32	M	3	51	4	21	56	27	Premature, Azathioprine, Tacrolimus
	45*	38	F (TW I)	3	150	6	26	341	230	Azathioprine
	46*	38	F (TW II)	3	132	5	26	267	287	Azathioprine
cut off TREC ≤10 KREC ≤6 n= 13 954	47	36	M	2	7	205	27	4	732	Premature, T-cell deficiency & B-cell defect of unknown cause
	48	34	M	2	91	4	27	133	106	Premature, Azathioprine, Tacrolimus
	49	28	M	2	9	12	n.a.			Premature, deceased
	50	38	M	2	47	4	25	231	176	
	51	42	F	2	139	3	28	315	326	
	52	32	F (TW II)	2	13	5	10	113	45	Premature
	53	24	F (TW II)	2	9	13	69	230	421	Premature
	54	32	M	2	88	6	55	224	342	Premature
	55	36	M	2	192	5	26	260	350	Premature
	56	24	M	2	9	79	64	12	441	Premature
	57	25	M	2	10	43	113	115	1221	Premature
	58#	25	M	2	24	5	n.a.			Premature, no presentation with infections as per available records
	59#	28	F	8	120	6	n.a.			Premature, Trisomy 21, no presentation with infections as per available records
	60	38	M	2	79	1	22	163	356	
	61	41	M	2	372	5	33	461	417	
	62	23	F (TW II)	2	7	11	17	53	16	Premature
	63	38	M	2	185	5	22	333	471	
	64§	40	F	4	109	6	20	209	50	Azathioprine

**siblings, §siblings, #declined further testing, n.a. = not available, M = male, F = female, BMT = bone marrow transplant, IgAD = IgA deficiency. TREC and KREC levels are expressed as number of copies/3.2mm dot.*

Table 5: Immunological investigation results for PID patients

	Age-matched reference range	**Patient 1** Artemis Deficiency	**Patient 2** Ataxia-telangiectasia	**Patient 3** Unknown genetic defect
Immunoglobulins				
IgA (g/L)	– 0.20 – 0.25	<0.06	<0.06	<0.08
IgG (g/L)	4.0 – 10.0 2.50 – 9.0	6.71	2.88*	3.0
IgM (g/L)	0.15 – 1.0	0.10*	0.34	0.10*
Lymphoctye subsets				
CD3+ T cells				
(x10^9/L)	2.3 – 7.0	<0.01*	0.54*	0.73*
(%)	60 – 85	1*	51*	23*
CD3+/CD4+ T cells				
(x10^9/L)	1.7 – 5.3	NA	0.39*	0.5*
(%)	41 – 68		37*	16*
CD3+/CD8+ T cells				
(x10^9/L)	0.4 – 1.7	NA	0.13*	0.18*
(%)	9 – 23		12	6*
CD4+/CD8+				
ratio	1.3 – 6.3	NA	3.08	2.8
CD19+ B cells				
(x10^9/L)	0.6 – 1.9	<0.01*	0.09*	1.73
(%)	4 – 26	<0.5*	8	52*
CD16+/CD56+ NK cells				
(x10^9/L)	0.2 – 1.4	0.39	0.48	0.66
(%)	3 – 23	90*	41*	20

** denotes result outside of the age-matched reference range, NA = not analysed*

CHAPTER 5: APPLICATION OF DNA-BASED SCREENING METHODOLOGIES BEYOND THE NEWBORN PERIOD

5.1 Introduction and Contextual Statement

In newborn screening programs, the quantitation of TREC and KREC has been demonstrated to be an effective method by which to identify infants with severe forms of PID manifested by T and/or B cell lymphopaenia. However, there is limited data regarding the utility of these assays in the diagnostic evaluation of patients with suspected PID beyond the newborn period. Chapter 5 examines the role of TREC/KREC assays beyond the newborn period, particularly in the context of XLA and XLA-like disease.

5.2 Publication: Kappa Recombining Excision Circle Levels Remain Low or Absent Throughout Life in Patients with X-Linked Agammaglobulinemia

This chapter presents the third publication included in this thesis. The following paper, entitled 'Kappa recombining excision circle levels remain low or absent throughout life in patients with X-linked agammaglobulinemia', by Jovanka King, Stephan Borte, Nicholas Brodszki, Ulrika von Döbeln and Lennart Hammarström was published in the peer reviewed journal, Paediatric Allergy and Immunology, in March 2018 (2018:1-4, doi.org/10.1111/pai.12893).

ARTICLE METRICS (AS AT 11 JANUARY 2019):

Journal Impact Factor: 4.137

Statement of Authorship

Title of Paper	KREC levels remain low or undetectable throughout life in patients with X-linked agammaglobulinemia.
Publication Status	Published
Publication Details	KREC levels remain low or undetectable throughout life in patients with X-linked agammaglobulinemia. King J, Borte S, Brodszki N, von Döbeln U, Smith CIE, Hammarström L. Pediatr Allergy Immunol. 2018 Mar 15. doi: 10.1111/pai.12893. [Epub ahead of print] PMID: 29543351

Principal Author

Name of Principal Author (Candidate)	Dr Jovanka King		
Contribution to the Paper	Study design, writing of ethics application, laboratory work (processing of samples), database establishment, data collection, data analysis, writing of manuscript, production of tables and figures.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	11/4/18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Professor Lennart Hammarström		
Contribution to the Paper	Conceptualised & established study design, critical review of manuscript.		
Signature		Date	2018-04-11

Title of Paper	KREC levels remain low or undetectable throughout life in patients with X-linked agammaglobulinemia.
Publication Status	Published
Publication Details	KREC levels remain low or undetectable throughout life in patients with X-linked agammaglobulinemia. King J, Borte S, Brodszki N, von Döbeln U, Smith CIE, Hammarström L. Pediatr Allergy Immunol. 2018 Mar 15. doi: 10.1111/pai.12893. [Epub ahead of print] PMID: 29543351

Name of Co-Author	Dr Stephan Borte		
Contribution to the Paper	Laboratory work (processing of samples), review of manuscript.		
Signature		Date	2018-04-29

Title of Paper	KREC levels remain low or undetectable throughout life in patients with X-linked agammaglobulinemia.
Publication Status	Published
Publication Details	KREC levels remain low or undetectable throughout life in patients with X-linked agammaglobulinemia. King J, Borte S, Brodszki N, von Döbeln U, Smith CIE, Hammarström L. Pediatr Allergy Immunol. 2018 Mar 15. doi: 10.1111/pai.12893. [Epub ahead of print] PMID: 29543351

Name of Co-Author	Dr Nicholas Brodzski		
Contribution to the Paper	Recruitment of patients & provision of patient samples, review of manuscript.		
Signature		Date	2018 04 12

Title of Paper	KREC levels remain low or undetectable throughout life in patients with X-linked agammaglobulinemia.
Publication Status	Published
Publication Details	KREC levels remain low or undetectable throughout life in patients with X-linked agammaglobulinemia. King J, Borte S, Brodszki N, von Döbeln U, Smith CIE, Hammarström L. Pediatr Allergy Immunol. 2018 Mar 15. doi: 10.1111/pai.12893. [Epub ahead of print] PMID: 29543351

Name of Co-Author	Dr Ulrika von Döbeln		
Contribution to the Paper	Provision of patient samples, review of manuscript.		
Signature		Date	2018-04-12

Title of Paper	KREC levels remain low or undetectable throughout life in patients with X-linked agammaglobulinemia.
Publication Status	Published
Publication Details	KREC levels remain low or undetectable throughout life in patients with X-linked agammaglobulinemia. King J, Borte S, Brodszki N, von Döbeln U, Smith CIE, Hammarström L. Pediatr Allergy Immunol. 2018 Mar 15. doi: 10.1111/pai.12893. [Epub ahead of print] PMID: 29543351

Name of Co-Author	Professor CI Edvard Smith		
Contribution to the Paper	Recruitment of patients & provision of patient samples, review of manuscript.		
Signature		Date April 12, 2018	

DOI: 10.1111/pai.12893

LETTER TO THE EDITOR

WILEY

Kappa-deleting recombination excision circle levels remain low or undetectable throughout life in patients with X-linked agammaglobulinemia

To the Editor,

X-linked agammaglobulinemia (XLA) is an inborn antibody deficiency syndrome, resulting from mutations in the *BTK* gene which give rise to a maturational arrest in B cell development at the pre-B cell stage and subsequent absence of peripheral B lymphocytes, hypogammaglobulinemia and ensuing severe and recurrent infections.[1,2] Early diagnosis and treatment with immunoglobulin replacement therapy are essential to prevent sequelae of chronic, untreated infections.[3]

It has previously been demonstrated that XLA can be diagnosed soon after birth through kappa-deleting recombination excision circle (KREC) level quantification.[4,5] KREC are circular, episomal pieces of DNA which are produced during V(D)J recombination within the *IGK* locus of B lymphocytes.[6] As such, KREC are a surrogate marker of bone marrow output of B lymphocytes and can be quantified using real-time quantitative polymerase chain reaction (qPCR) techniques.[4,6] KREC levels have previously been shown to be absent or markedly reduced in the newborn dried blood spots (DBS) of patients with XLA, whereas T cell receptor excision circle (TREC) levels are similar to those of healthy controls.[4,5] However, the utility of the KREC assay as a diagnostic tool for XLA in a clinical setting outside of neonatal screening programs has not previously been evaluated. We aimed to quantify serial KREC levels in patients with XLA at three time points: at birth; the time of diagnosis; and the current day to evaluate the trend over time and to determine the clinical utility of the KREC assay in the diagnosis of XLA beyond the newborn period.

Twelve male patients with XLA and one female patient with an autosomal recessive, XLA-like disease were recruited from three clinical centers in Sweden. Thirteen healthy adult and storage time-matched newborn control specimens were also included in the study. The regional ethical board in Stockholm approved the study (ethical permit 2016/189-31/2). Written, informed consent was obtained from all participants in accordance with the ethical standards of the Helsinki declaration and amendments.

Where available, DNA samples were obtained for each patient at birth (extracted from DBS from archived Guthrie cards), time of diagnosis, and the current day. DNA was extracted from whole blood and DBS specimens using our previously described method.[4] KREC, TREC, and ACTB (β-actin) levels were quantified using a multiplexed qPCR method, as previously described.[4]

All 13 patients had hypogammaglobulinemia and absent or markedly reduced B lymphocyte counts quantitated by flow cytometry, and a causative mutation was identified in all cases (Table 1). KREC levels were markedly reduced in all patients at birth, the time of diagnosis, and the current day compared with healthy control subjects (Figure 1A). In most cases, TREC levels in the XLA patients were comparable to those in healthy control subjects, although levels were noted to be higher in a few cases (Figure 1B). Although TREC levels typically decline with increasing age, there are significant interindividual differences in TREC levels both in newborns and across the age spectrum, and therefore, this likely reflects biologic variation.

Patients with XLA and XLA-like disease are expected to present in the first year of life, corresponding to waning protective maternal immunoglobulin levels at 6-9 months of age. However, diagnosis is frequently delayed. In a study of 73 patients with XLA, the mean age of diagnosis was 3.5 years.[3] In our cohort, only 2 of 13 patients were diagnosed in the first year of life, whereas the diagnosis was significantly delayed (up to 6 years of age) in the remainder of cases. The association between delayed diagnosis and an increased rate of disease complications is well described, with evidence suggesting that outcomes are improved with early institution of immunoglobulin replacement therapy.[3] Our results suggest that a newborn screening program incorporating a KREC assay would have successfully identified these patients at birth, thereby facilitating early institution of therapy and prevention of complications. These results reinforce our previous findings demonstrating that a KREC assay will successfully identify patients with XLA in the newborn period.[4] In addition, we have demonstrated that this assay also enables identification of newborns with autosomal recessive XLA-like disease. Some patients with XLA or XLA-like disease are described to have a "leaky" phenotype, resulting in production of very small numbers of poorly functional B lymphocytes. In our cohort, despite minor KREC level fluctuations observed in some individual patients, these remained well below observed levels in healthy control subjects, suggesting that even those patients with a leaky phenotype will still be accurately identified using a KREC assay.

In addition to the identification of patients with congenital B cell defects, newborn screening protocols incorporating multiplexed TREC/KREC assays offer many advantages over TREC screening alone. This includes improved characterization of patients with SCID and identification of a variety of other conditions associated with immunodeficiency including Nijmegen breakage syndrome, late onset ADA deficiency, and ataxia telangiectasia.[4] KREC

TABLE 1 Characteristics of included patients

Patient	Diagnosis	Gene	Mutation[a]	Age at diagnosis	Current age	IgA (mg/dL)	IgG (mg/dL)	IgM (mg/dL)	White blood cells (×10^9/L)	Lymphocytes (×10^9/L)	CD19+/CD20+ cells (×10^9/L)	CD19+/CD20 + cells (%)
1	XLA	*BTK*	c.309 + 602G > T, p.Val104delins ESKNYKFGLQFGLLTS[b]	8 mo	43 y	<0.02	1.2	<0.3	4.2	1.4	1.4	0
2	XLA	*BTK*	c.776 + 1G > A	4 y	36 y	0	10.4[b]	0	3.9	1.4	0	0
3	XLA	*BTK*	c.1383_1384delTG, p.Tyr461X	8 mo	28 y	0	5.75[b]	0	6.3	1.5	0	0
4	XLA	*BTK*	c.1922G > A, p.Arg641His	2 y	34 y	0	10.5[b]	0	5.2	NA	1	0
5	XLA	*BTK*	c.1236G > A, p.Trp421X	3 y	44 y	0	5.8[b]	0.1	9.9	1.2	0.01	1
6	XLA	*BTK*	c.1574G > A, p.Arg525Gln	5 y	37 y	0	8.92[b]	0	3.8	1.7	0	0
7	XLA	*BTK*	c.1631G > C, p.Arg544Thr	4 y	42 y	<0.06	1.57	0.5	5.7	1.66	0	0
8	XLA	*BTK*	c.1905_1906insTTTTAG, p.His635_Glu636insPheX	2 y	35 y	<0.04	<0.2	<0.06	3.7	1	0	0
9	XLA	*BTK*	c.1684C > T, p.Arg562Trp	20 mo	15 y	0.1	<0.39	0.2	9.1	2.9	<10	<1
10	XLA	*BTK*	c.82C > T, p.Arg28Cys	6 y	18 y	<0.02	0.6	<0.02	20	4.2	0	0
11	XLA	*BTK*	c.587_588 + 1del, p.G196Hfs[b]9	20 mo	4 y	<0.07	<0.33	0.81	10.2	5.4	<10	<1
12	XLA	*BTK*	c.82C > T, p.Arg28Cys	3 y	16 y	<0.02	0.6	0.58	14.8	3.2	0	0
13	XLA-like	*IGGL1*	c.258delG, p.G86 fs	4 y	12 y	0.62	3.9[b]	0.16	7	3.2	20	<1

XLA, X-linked agammaglobulinemia; *BTK*, Bruton's tyrosine kinase; *IGLL1*, Immunoglobulin lambda like polypeptide 1; NA, not available.
[a]Previously published mutations (http://structure.bmc.lu.se/idbase/BTKbase/).
[b]Levels measured during immunoglobulin replacement therapy.

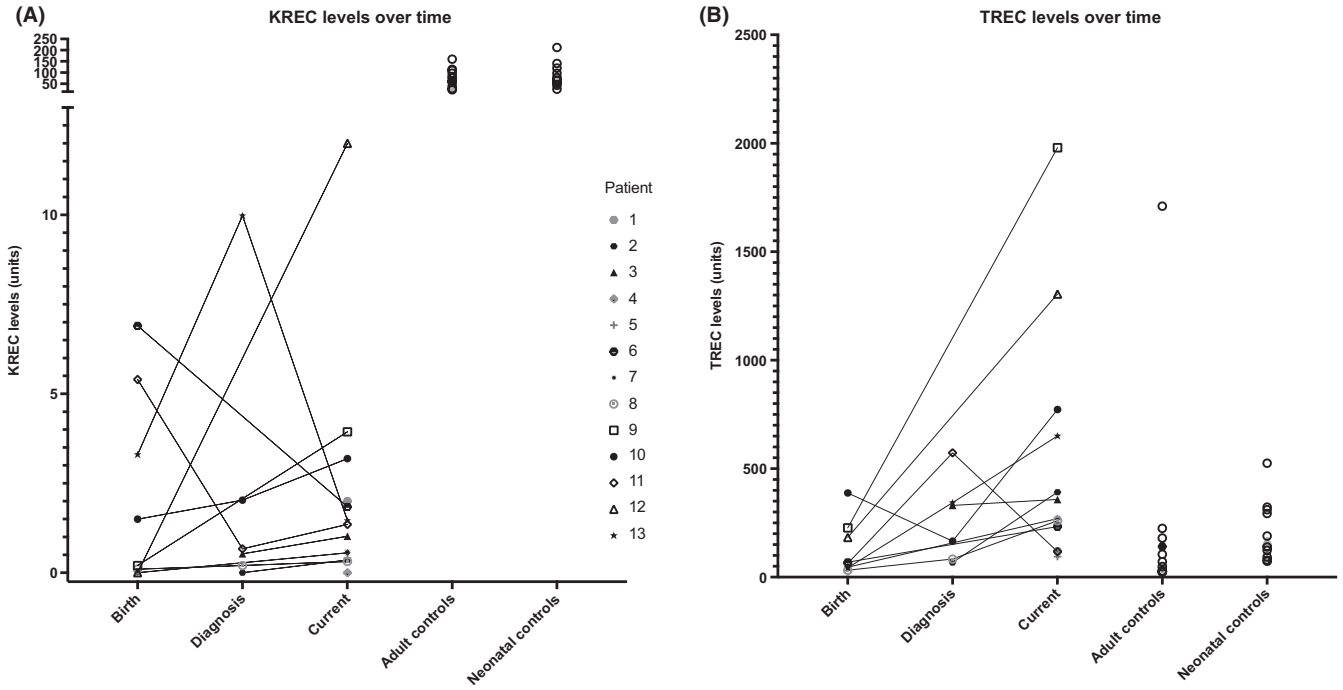


FIGURE 1 Kappa-deleting recombination excision circle (KREC) (A) and T-cell receptor excision circle (TREC) (B) levels in patients with X-linked agammaglobulinemia (XLA) at three time points, and in healthy adult and neonatal controls. Solid lines demonstrate the trend in levels for individual patients from samples collected at different time points

level estimation has also been useful in other contexts in addition to newborn screening, including the assessment of immune reconstitution following hematopoietic stem cell transplantation.[6] Our data suggest that KREC level estimation is a useful diagnostic test for investigation of patients with suspected congenital B cell deficiency disorders beyond the newborn period. It may be an adjunct or alternative to FACS-based B lymphocyte enumeration. KREC analysis may be used to verify FACS findings, or used in lieu of FACS should this not be readily available. KREC analysis may be more cost-effective and also confers some technical advantages including the ability to run the assay on stored DNA or DBS samples, which can easily be transported between laboratories and negates the need for a fresh blood sample for testing.

We quantified serial KREC levels in a cohort of patients with XLA and demonstrated that these are markedly reduced or absent at birth and remain consistent over time. The same pattern was noted in one patient with XLA-like disease. Thus, we have reinforced the diagnostic utility of the KREC assay in identifying patients with XLA and other forms of congenital B-cell deficiency at birth and beyond the newborn period.

We thank the patients and their families for participating in this study and also Susanne Hansen (Immunodeficiency Unit at the Karolinska University Hospital Huddinge) and Jacek Winiarski (at the Department of Pediatrics at Karolinska University Hospital Huddinge) for providing clinical information on selected patients. This work was supported by grants from Vetenskapsrådet and Avtal om Läkarutbildning och Forskning to Prof Lennart Hammarström, and from Vetenskapsrådet to Prof C. I. Edvard Smith. Dr Jovanka King acknowledges the support of the RCPA Foundation (Mike and Carole Ralston Travelling Fellowship 2016).

CONFLICT OF INTEREST

L.H. and S.B have stock in ImmunoIVD (previously Mabtech Diagnostics) a company that has commercialized a kit for TREC/KREC screening. However, data for this study were generated using the original manual method.[4]

FUNDING INFORMATION

This work was supported by grants from Vetenskapsrådet and the Stockholm County Council (ALF project) to Lennart Hammarström, and from Vetenskapsrådet to C. I. Edvard Smith.

ORCID

Jovanka King http://orcid.org/0000-0002-6783-4830

Jovanka King[1,2]
Stephan Borte[1,3]
Nicholas Brodszki[4]
Ulrika von Döbeln[5,6]
C. I. Edvard Smith[7]
Lennart Hammarström[1,8]

[1]*Division of Clinical Immunology, Department of Laboratory Medicine, Karolinska University Hospital Huddinge, Stockholm, Sweden*

[2]*Department of Immunopathology, SA Pathology, Robinson Research Institute and Discipline of Paediatrics, School of Medicine, University of Adelaide, North Adelaide, South Australia*

[3]*ImmunoDeficiencyCenter Leipzig (IDCL) at Hospital St. Georg gGmbH Leipzig, Leipzig, Germany*

[4]*Department of Pediatrics, Children's Hospital Lund, Skåne University Hospital, Lund, Sweden*

[5]*Centre for Inherited Metabolic Diseases, Karolinska University Hospital Solna, Stockholm, Sweden*

[6]*Department of Medical Biochemistry and Biophysics, Division of Molecular Metabolism, Karolinska Institutet, Stockholm, Sweden*

[7]*Clinical Research Center, Department of Laboratory Medicine, Karolinska University Hospital Huddinge, Stockholm, Sweden*

[8]*BGI-Shenzhen, Shenzhen, China*

Email: lennart.hammarstrom@ki.se

REFERENCES

1. Vetrie D, Vorechovsky I, Sideras P, et al. The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. *Nature*. 1993;361:226-233.
2. Bruton OC. Agammaglobulinemia. *Pediatrics*. 1952;9:722-728.
3. Plebani A, Soresina A, Rondelli R, et al. Clinical, immunological, and molecular analysis in a large cohort of patients with X-linked agammaglobulinemia: an Italian multicenter study. *Clin Immunol*. 2002;104:221-230.
4. Borte S, von Döbeln U, Fasth A, et al. Neonatal screening for severe primary immunodeficiency diseases using high-throughput triplex real-time PCR. *Blood*. 2012;119:2552-2555.
5. Nakagawa N, Imai K, Kanegane H, et al. Quantification of kappa-deleting recombination excision circles in Guthrie cards for the identification of early B-cell maturation defects. *J Allergy Clin Immunol*. 2011;128:223-225.e2.
6. van Zelm MC, Szczepanski T, van der Burg M, van Dongen JJ. Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. *J Exp Med*. 2007;204:645-655.

CHAPTER 6: A TRANSCRIPTOMIC APPROACH TO NEWBORN SCREENING FOR HYPOGAMMAGLOBULINAEMIA

6.1 Contextual Statement

Currently available newborn screening strategies for primary immunodeficiency diseases enable detection of conditions manifested by T and/or B cell lymphopaenia. Several forms of PID are associated with hypogammaglobulinaemia, and thus screening for immunoglobulin deficiency provides an opportunity to increase our capacity to identify a broader range of diseases. In this chapter, preliminary results from a small pilot investigation are presented, evaluating a transcriptomic approach to the identification of children with hypogammaglobulinaemia with potential application as a newborn screening test. This chapter is presented in the form of an unpublished manuscript.

Statement of Authorship

Title of Paper/Chapter	A Transcriptomic Approach to Newborn Screening for Hypogammaglobulinaemia.
Publication Status	Unpublished and unsubmitted work written in manuscript style
Publication Details	Unpublished data from pilot study undertaken during candidature, presented in manuscript format.

Principal Author

Name of Principal Author (Candidate)	Dr Jovanka King		
Contribution to the Paper	Ethics & Research Governance application preparation & submission, patient recruitment, sample collection, background research & literature review, development of laboratory assays, laboratory work, quality control, trouble shooting, data analysis, manuscript preparation.		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	22/8/2018

6.2 Introduction

Immunoglobulin deficiency disorders constitute the largest group of primary immunodeficiency disorders[1]. In addition, hypogammaglobulinaemia is a feature of other forms of PID, including severe combined immunodeficiency (SCID) and other combined immunodeficiencies (CID)[1].

Maternal immunoglobulin transfer, particularly in the last trimester of pregnancy, is an important factor contributing to the immunity of the infant, whose own endogenous immunoglobulin production increases over time with advancing maturity[22]. Due to the presence of maternal Immunoglobulin G (IgG) and Immunoglobulin A (IgA) in the cord blood and neonatal circulation, it is not possible to reliably quantify endogenous production of either protein in infants using standard techniques such as nephelometry on serum samples, nor on eluates from dried blood spots[20].

There are well over 300 genetic causes of PID, and currently available newborn screening strategies only enable a small number of these conditions to be detected[32]. Newborn screening for severe forms of PID manifested by T and/or B cell lymphopaenia using TREC and KREC assays have been well described, and are used in prospective screening programs and studies in many countries[33]. Expansion of screening technologies to increase the number of screened diseases has included development of protein-based assays for the detection of granulocyte and complement deficiencies, targeted genetic sequencing and gene copy number assays[33]. However, there is no currently available strategy exists by which to screen infants for immunoglobulin deficiency. It is well documented that early detection of all forms of PID enables timely commencement of therapy and improved patient outcomes in terms of morbidity, mortality and quality of life[16].

Newborn screening cards are suitable for extraction and quantification of protein, DNA and RNA using various techniques[34,35]. RNA can be extracted from DBS specimens for analysis, and remains relatively stable for many years[35,36]. Karlsson *et al*. demonstrated that RNA can be successfully extracted from DBS at 1 month, 21 years and 27 years following collection, regardless of storage conditions (either at -4^{0}C or room temperature)[36]. Microarray technology on RNA extracted from DBS specimens to acquire genome-wide expression profiles has been described, supporting the notion that RNA can be successfully extracted and amplified from DBS specimens[34].

Immunoglobulin molecules are comprised of heavy and light chains, which are derived from genetic loci on chromosome 14 (heavy chain) and chromosome 2 (kappa light chain) and chromosome 22 (lambda light chain). Functional antibodies are formed as a result of recombination events in the variable, diversity and junctional regions (V(D)J), with increased affinity owing to later class switch recombination events and somatic hypermutation.

Expression of the immunoglobulin heavy chain genes has been evaluated in patients with CVID, with reduced transcript production noted, correlating with low serum immunoglobulin levels[37]. This suggests that assessment of gene expression may be one approach to identifying children with PID manifested by hypogammaglobulinaemia or agammaglobulinaemia, in the setting where protein cannot be reliably measured due to confounding factors such as maternal immunoglobulin contamination or gammaglobulin replacement therapy.

6.3 Aims and Hypotheses

The aim of this study was to:

1. Establish an effective method by which to extract RNA from DBS.
2. Develop an assay to evaluate expression of the Immunoglobulin G (*IGHG1*), A (*IGHA1*) and M (*IGHM*) heavy chain genes in healthy individuals and in patients with hypogammaglobulinaemia.
3. Determine the efficacy of this approach in identifying patients with hypogammaglobulinaemia and evaluate its applicability as a potential newborn screening test for PID manifested by hypogammaglobulinaemia.

It was hypothesised that:

1. RNA can be effectively extracted from DBS for downstream use.
2. Expression of the *IGHG1*, *IGHA1* and *IGHM* genes will be reduced in patients with hypogammaglobulinaemia compared with healthy control subjects.
3. This testing strategy can be applied as a newborn screening testing strategy to enable identification of infants with PID manifested by hypogammaglobulinaemia.

6.4 Materials and Methods

Ethical considerations

This study was approved by the Women's and Children's Hospital Network Human Ethics Research Committee (HREC/16/WCHN/164) and Research Governance Committee (SSA/16/WCHN/192). Written, informed consent was obtained from all participants. This research was conducted in accordance with the Helsinki Declaration of 1964 and its later amendments.

Patient and sample recruitment

Nineteen paediatric patients with PID, including five patients with X-linked agammaglobulinaemia (XLA) and one with X-linked hyper IgM syndrome (X-HIGM) were

recruited from the immunology department in a single South Australian centre. Characteristics of included patients are provided in Table 6.5.1. The majority of these patients were currently hypogammaglobulinaemic based on nephelometry results, and/or were receiving either intravenous or subcutaneous gammaglobulin replacement therapy. In addition, 13 healthy adult control subjects were recruited. Blood was collected from each participant onto filter paper, mimicking dried blood spots collected on Guthrie cards in the neonatal period. The original Guthrie card specimen for one XLA patient (Patient 5) was also accessed. Fifteen anonymised dried blood spot samples, up to 17 years of age, were accessed from the SA Neonatal Screening Laboratory, Adelaide.

RNA extraction and cDNA synthesis

Two 3.2mm punches were removed from each DBS sample for each specimen. RNA was extracted from the DBS punches using a method we developed. Briefly, the DBS punches were eluted in 200μL of rapid RNA extraction solution (Ambion, Applied Biosystems, MA, USA), and incubated at room temperature on a plate shaker for 15 minutes. The eluate was removed, and RNA and DNA was extracted using the QiaAMP MinElute Virus Spin Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesised using the Bio-Rad cDNA iScript synthesis kit (Bio-Rad, California, USA). Twenty nanograms of RNA was added to 4μL of 5X iScript Reaction Mix, 1μL of iScript Reverse Transcriptase and nuclease-free water to give a final volume of 20μL. Thermal cycler conditions were as follows: Priming 25^0C for 5 minutes, reverse transcription 46^0C for 20 minutes, RT inactivation 95^0C for 1 minute, hold 4^0C for 20 minutes.

Gene expression analysis

Five microlitres of the cDNA product, diluted 1:5, was added to 10μL of 2X Taqman Gene Expression Mastermix (Applied Biosystems, MA, USA), 7μL of RNAse-free water and 1μL of 10X TaqMan gene expression assay for one of the following per reaction: *IGHG1* (Hs00378340_m1, Cat. No. 4331182, FAM-MGB), *IGHA1* (Hs00733892_m1, Cat. No. 4331182, FAM-MGB), *IGHM* (Hs00378435_m1, Cat. No. 4331182, FAM-MGB) and *ACTB* (Hs01060665_g1, Cat. No. 4331182, FAM-MGB) (Applied Biosystems, MA, USA). *ACTB* (β-actin) was used as a housekeeper gene. Samples were run in triplicate for the genes of interest and housekeeper gene, with appropriate positive and negative (non-template) controls on a Bio-Rad iQ5 qPCR instrument (Bio-Rad, California, USA). Thermal cycler conditions were as follows: hold 50^0C for 2 minutes, hold 95^0C for 10 minutes followed by 70 X cycles (95^0C for 15 second, 60^0C for 1 minute), standard ramp rate. Quality control was maintained by assessment of a minimum C_T threshold for *ACTB* for each sample, and samples achieving

threshold at a C_T of $\geq$45 with poor traces were considered non-specific. Given that the RNA used was derived from DBS and was therefore of a lower concentration and quality compared with RNA extracted from other sources (such as fresh whole blood or purified PBMCs), later than usual amplification was noted for *ACTB* and other genes assessed.

Data analysis

Data was analysed using the ΔC_T method, comparing the gene of interest with the housekeeper gene for each patient, and comparing these with pooled healthy control values to determine relative gene expression. Statistical analysis was not performed due to the low number of samples and heterogeneity of patient groups. Figures were generated using GraphPad Prism software, Version 7.0d (California, USA).

6.5 Results

Characteristics of included patients

Clinical characteristics and laboratory investigation results of patients included in this study are presented in Table 1.

Table 1: Characteristics of included patients

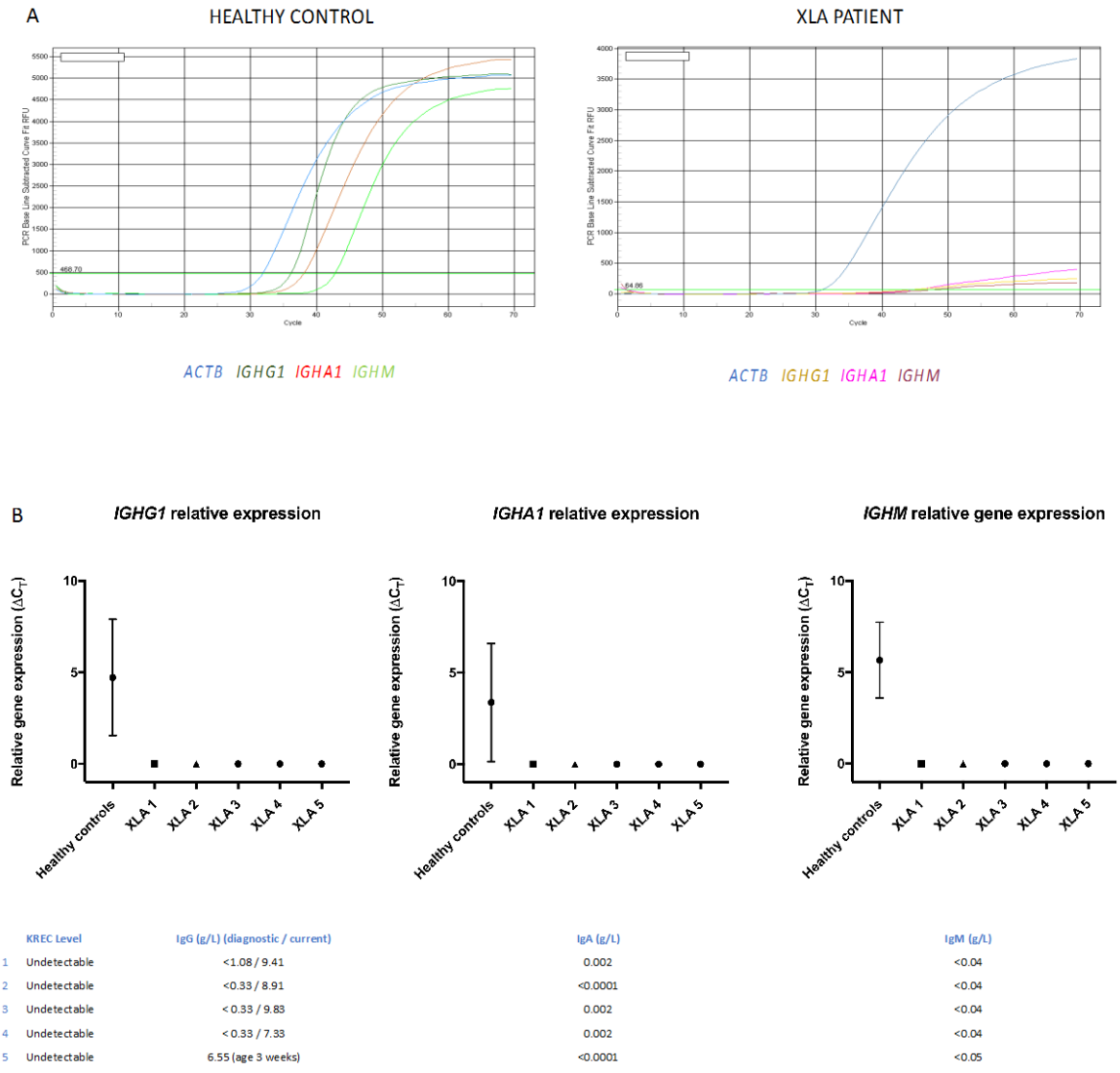
Patient	Identifier	Age	Clinical Diagnosis	Gene	Mutation	IgG (g/L) (diagnosis)	IgG (g/L) (current)	IgA (g/L)	IgM (g/L)	CD19+ cells (x10^3/uL)
1	XLA1	2y	XLA	*BTK*	c.957_958delGT	<0.03	7.73*	0.002	0.11	0
2	XLA2	10y	XLA	*BTK*	c.1750+5G>A	<0.33	8.31*	<0.0001	<0.04	0
3	XLA3	17y	XLA	*BTK*	c.994C>T	1.08	9.41*	0.002	<0.04	0.06
4	XLA4	2y	XLA	*BTK*	c.[557del];[0]	0.29	9.26*	<0.001	0.04	<0.04
5	XLA5	3w	XLA	*BTK*	c.957_958delGT	6.51**	6.51*	<0.0001	<0.05	0
6	HIGM	16y	HIGM	*CD40L*	Xq26.3 del	<0.33	5.6*	<0.05	0.53	2.82
7	APDS1	14y	APDS	*PI3K*	c.3061G>A	10.2	23.7*	0.94	2.14	0.2
8	APDS2	18y	APDS	*PI3K*	c.3061G>A	11.8	16.1*	0.71	3.67	0.12
9	TS21 + SAD	10y	Trisomy 21 / SAD	*T21*	47,XY, +21	9.94	10.8*	1.81	0.29	0.05
10	22q	13y	22q deletion	*22q*	XX.ish del (22)(q11.2q11.2)	6.23	10.3	1	0.17	1.27
11	THI	4y	THI	*N/A*		2.43	3.47*	0.54	0.82	2.13
12	ARPC1B	7y	ARPC1B deficiency	*ARPC1B*	c.64+2T>A	12.4	14.4*	10.51	0.62	0.69
14	CVID1	10y	CVID	*N/A*		4.71	9.89*	0.92	0.47	0.62
13	CVID2	9y	CVID	*N/A*		3.4	8.74*	0.65	0.66	0.62
15	CVID3	11y	CVID	*N/A*		3.49	6.87*	0.44	0.52	0.25
16	CVID4	11y	CVID	*N/A*		4.67	8.92*	0.74	0.8	0.21
17	CVID5	16y	CVID	*N/A*		4.98	5.57*	0.52	0.72	0.39
18	CVID6	15y	CVID	*N/A*		4.65	9.45*	0.26	0.01	0.31
19	CVID7	13y	CVID	*N/A*		5.03	7.81*	0.36	0.85	0.25

** receiving immunoglobulin replacement therapy, ** IgG level reflecting maternal immunoglobulin level*

XLA = X-linked agammaglobulinaemia, HIGM = X-linked hyper-immunoglobulin M syndrome, APDS = activated PI3K (phosphoinositide-3-kinase) delta syndrome, SAD = specific antibody deficiency, T21 = Trisomy 21, 22q = 22q11.2 deletion syndrome, THI = transient hypogammaglobulinaemia of infancy, CVID = common variable immunodeficiency

Immunoglobulin gene expression was absent in patients with XLA

Five patients with XLA were included in this analysis, and all had absent expression of *IGHG1*, *IGHA1* and *IGHM*, in the context of detectable *ACTB* expression (Figure 1 A & B). Patient 5 was recruited as a newborn infant (sibling of patient 1) whose original Guthrie card was sampled for extraction of RNA. For this patient, immunoglobulin levels were measured at 3 weeks of life and this value therefore reflects the maternal IgG level (Table 1). KREC levels were undetectable in all five patients.

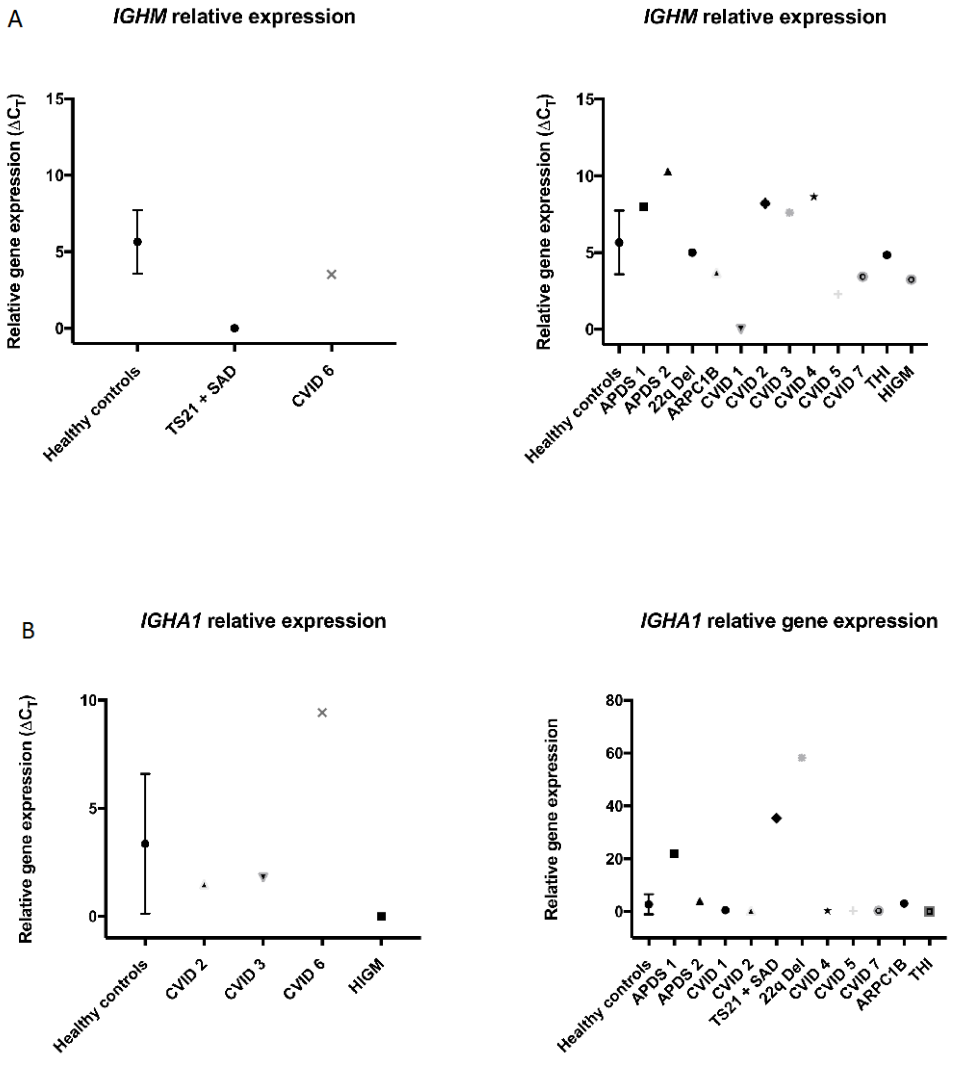


	KREC Level	IgG (g/L) (diagnostic / current)	IgA (g/L)	IgM (g/L)
XLA 1	Undetectable	<1.08 / 9.41	0.002	<0.04
XLA 2	Undetectable	<0.33 / 8.91	<0.0001	<0.04
XLA 3	Undetectable	< 0.33 / 9.83	0.002	<0.04
XLA 4	Undetectable	< 0.33 / 7.33	0.002	<0.04
XLA 5	Undetectable	6.55 (age 3 weeks)	<0.0001	<0.05

Figure 1: Immunoglobulin heavy chain (*IGHG1*, *IGHA1*, *IGHM*) and *ACTB* expression in patients with XLA. A) Representative qPCR curves from one healthy control and one patient with XLA. B) Relative gene expression for all XLA patients compared with healthy control subjects (ΔCt) and corresponding KREC and immunoglobulin protein results (measured by nephelometry)

Immunoglobulin gene expression is variable amongst healthy controls, PID patients with normal or partial reductions in levels of IgM, IgA and IgG

Expression of *IGHM, IGHA1* and *IGHG1* was variable between healthy controls and patients with PID who had either normal or partial reduction in their immunoglobulin levels (Figure 2). *IGHG1* expression was reduced in most patients with low IgG levels (prior to commencing immunoglobulin therapy). *IGHG1* was expressed in one patient with 22q deletion syndrome, one with APRC1B deficiency who had normal immunoglobulin levels measured by nephelometry, and in three patients with CVID.



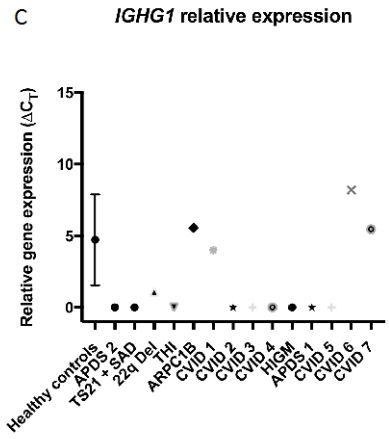


Figure 2: *IGHM*, *IGHA1* and *IGHG1* expression (∆Ct) in healthy control subjects and patients with PID with normal or decreased levels of IgA and IgM as measured by nephelometry. A) Relative expression of *IGHM* in controls and PID patients with normal (left panel) and low (right panel) IgM levels. B) Relative expression of *IGHA1* in controls and PID patients with normal (left panel) and low (right panel) IgA levels. C) Relative expression of *IGHG1* in controls and PID patients.

RNA can be extracted from DBS stored for up to 17 years and used for gene expression assays

Using our method, RNA could be effectively extracted and ACTB expression was detectable from neonatal DBS sampled from Guthrie cards aged between 3 weeks and 18 years of age (Figure 3 (A)).

Immunoglobulin gene expression is detectable in newborn DBS specimens

Immunoglobulin heavy chain gene expression was detectable in recently collected DBS from newborn Guthrie card specimens. *IGHA1* and *IGHM* were more highly expressed than *IGHG1* (Figure 3 (B)).

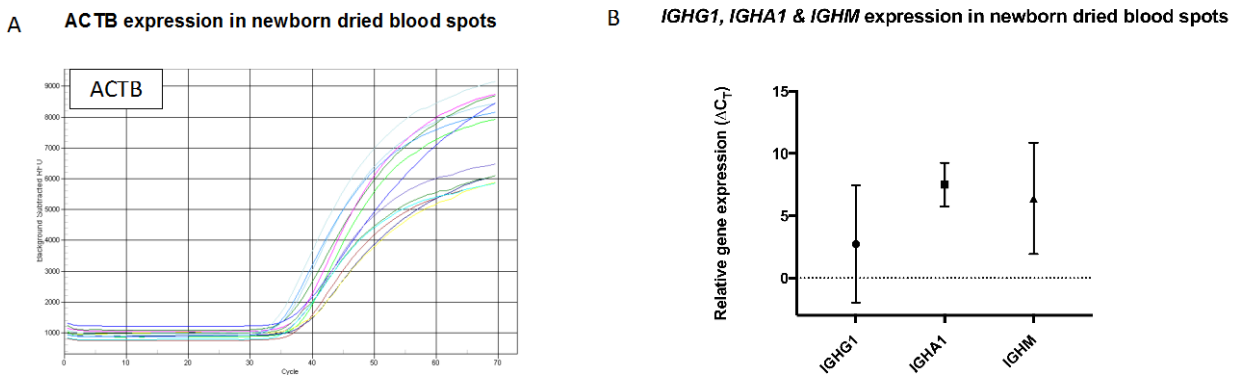


Figure 3: A) *ACTB* expression in neonatal DBS specimens stored at room temperature for up to 17 years. B) Expression of *IGHG1*, *IGHA1* and *IGHM* from recently collected neonatal DBS specimens (n=10) (ΔCt).

6.6 Discussion

Despite recent technological advances in methodologies for screening for PID, at the current time, it is not possible to screen newborns for PID manifested by hypogammaglobulinaemia. Here, we describe a novel assay by which RNA extracted from DBS specimens can be used to evaluate immunoglobulin heavy chain gene expression as a surrogate marker for production of IgG, IgA and IgM.

Our results indicate that this technique is effective in identifying children with absolute deficiencies of immunoglobulin production, as is the case in patients with XLA, where *IGHG1*, *IGHA1* and *IGHM* expression are absent. We demonstrated on an original Guthrie card specimen that this technique could accurately identify a newborn with XLA, providing proof of concept of this method as a potentially useful newborn screening test.

We also found that the degree of immunoglobulin heavy chain gene expression is highly variable between healthy control individuals, and amongst children with PID with either normal or low immunoglobulin levels measured by nephelometry. Given this, our technique in its current format is not sufficiently sensitive to identify partial reductions in immunoglobulin levels.

Although it has previously been demonstrated that RNA can be extracted from DBS[34-36,38], this has classically been challenging, due to RNA degradation over time. We demonstrated that our method for RNA extraction from DBS specimens yielded RNA of sufficient quality and quantity for downstream applications. This was effective even for DBS stored at room

temperature for up to 17 years. We also demonstrated, in a small number of neonatal Guthrie card DBS specimens, that the immunoglobulin heavy chain genes are expressed early in life. *IGHA1* and *IGHM* were more highly expressed than *IGHG1*, which correlates with our current understanding that IgG production increases incrementally during early infancy[22]. Based on the results of our small pilot results, the combination of reduced or absent expression of all three genes simultaneously is suggestive of a severe form of antibody deficiency (i.e. XLA). Our one patient with X-HIGM syndrome also had absent *IGHA1* and *IGHG1* expression, but preserved *IGHM* gene expression.

Current screening technologies applying a multiplexed TREC/KREC assay is able to identify patients with XLA[10], and hence our current transcriptomic assay would not confer any additional benefit over this assay for detection of hypogammaglobulinaemia secondary to congenital B cell deficiencies. However, absent expression of *IGHA1* and *IGHG1* in our patient with X-HIGM, with preserved *IGHM* expression, suggesting that this severe form of antibody deficiency with normal KREC levels[10] is detectable using our assay. Hence, this assay shows potential to increase the breadth of screened immunodeficiency diseases.

A limitation of this pilot study is the small sample size. Performing this assay on a larger number of patients and healthy controls, and performing correlation studies with nephelometry results will provide a better indication of its sensitivity and specificity. It is possible that this assay could be further optimised to improve testing characteristics. In order to apply this assay as a newborn screening test, this method requires evaluation on a large cohort of neonates to determine expected 'normal ranges' for gene expression in order to identify infants with abnormal results. The kinetics of immunoglobulin heavy chain gene expression over time during the early neonatal period and infancy could also be explored. Expression of the *IGHD* gene, encoding for IgD production, could also be assessed in follow-up studies as it may also be a useful marker for significant immunoglobulin deficiency states.

Once further developed, this gene expression assay may have future applications beyond the newborn period. This assay may be useful as an adjunctive testing strategy in the work-up of patients with possible PID, and may also provide useful clinical information regarding an individual's current capacity to produce their own endogenous immunoglobulins whilst receiving gammablobulin replacement therapy. This would be particularly useful for informing clinical decisions regarding optimal timing for a trial of cessation of replacement therapy, for example in the case of transient hypogammaglobulinaemia of infancy or in patients with secondary hypogammaglobulinaemia. In addition, this assay could be used as a more accurate estimate of endogenous immunoglobulin production in infants under 6 months, in whom maternal immunoglobulin G and A levels confound results obtained by nephelometry[20,22].

6.7 Conclusion

This early pilot data shows that using our method, RNA can be effectively extracted from DBS specimens and used in gene expression assays. This assay may be used to investigate the immunoglobulin transcriptome in health and disease. Expression of the *IGHM, IGHA1* and *IGHG1* genes is variable in healthy controls and PID patients with normal or reduced levels of IgA and IgM as determined by nephelometry. Our technique does, however, effectively identify patients with absolute deficiencies in antibody production such as XLA, who lack expression of all three genes simultaneously. We have demonstrated a proof of concept that this testing strategy can identify a newborn with XLA. It is anticipated that further refinement of this assay will enable identification of individuals with moderately reduced immunoglobulin levels, thereby becoming a strategy by which newborns can be screened for PID manifested by hypogammaglobulinaemia, with other potential applications beyond the newborn period.

6.8 Acknowledgement

I would like to acknowledge the contributions of Stephan Borte, Jezabel Varadé, David Shields, Alexander Quach, staff at the SA Neonatal Screening Laboratory, Michael Gold, Antonio Ferrante and Lennart Hammarström to the work presented in this chapter.

CHAPTER 7: POPULATION-BASED NEWBORN SCREENING MEETS PERSONALISED MEDICINE: IDENTIFYING SINGLE NUCLEOTIDE POLYMORPHISMS PREDICTING DISEASE SUSCEPTIBILITY

7.1 Introduction and Contextual Statement

Moving toward a modern healthcare model which aims to provide patients with personalised, precision medicine, it would be beneficial not only to diagnose disease states, but also to identify individuals at risk for developing specific diseases by screening for susceptibility factors. This is discussed in the context of screening neonates for genetic polymorphisms which confer susceptibility to specific infectious diseases. This chapter examines the role of genotyping-based assays as a potential tool to screen infants for single nucleotide polymorphisms in fucosyltransferase genes (*FUT2* and *FUT3*), enabling identification of their intrinsic susceptibility to a range of infections and other disease states. It further explores this approach as a means by which to apply personalised medicine.

7.2 Publication: Fucosyltransferase Gene Polymorphisms and Lewis b Negative Status are Frequent in Swedish Newborns, with Implications for Infectious Disease Susceptibility and Personalized Medicine

This chapter presents the fifth paper included in this thesis. The following paper, entitled **'**Fucosyltransferase gene polymorphisms and Lewis b negative status are frequent in Swedish newborns, with implications for infectious disease susceptibility and personalized medicine', by Jovanka King, Jezabel Varadé and Lennart Hammarström was accepted for publication in the peer reviewed journal, Journal of the Pediatric Infectious Diseases Society, on 7 August 2018. Supplementary material follows the paper.

ARTICLE METRICS (AS AT 11 JANUARY 2019):

Journal Impact Factor: 2.456

Statement of Authorship

Title of Paper	Fucosyltransferase gene polymorphisms and Lewis b negative status are frequent in Swedish newborns, with implications for infectious disease susceptibility and personalized medicine.
Publication Status	Accepted for Publication
Publication Details	The Journal of the Pediatric Infectious Diseases Society

Principal Author

Name of Principal Author (Candidate)	Dr Jovanka King
Contribution to the Paper	Literature review, study design, experimental design, laboratory work (processing of samples), result analysis, quality control, data analysis, statistical analyses, writing of manuscript, construction of tables and figures.
Overall percentage (%)	85%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 11/4/18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Professor Lennart Hammarström
Contribution to the Paper	Initiated concept, critical review of manuscript.
Signature	Date 2018-4-11

Title of Paper	Fucosyltransferase gene polymorphisms and Lewis b negative status are frequent in Swedish newborns, with implications for infectious disease susceptibility and personalized medicine.
Publication Status	Submitted for Publication
Publication Details	Submitted to the Journal of the Pediatric Infectious Diseases Society

Name of Co-Author	Dr Jezabel Varadé		
Contribution to the Paper	Assistance & supervision of experimental & study design, laboratory work & data/statistical analysis, critical review of manuscript.		
Signature		Date	2018-04-12

Journal of the Pediatric Infectious Diseases Society

ORIGINAL ARTICLE

Fucosyltransferase Gene Polymorphisms and Lewis^b-Negative Status Are Frequent in Swedish Newborns, With Implications for Infectious Disease Susceptibility and Personalized Medicine

Jovanka R. King,[1,2] Jezabel Varadé,[1] and Lennart Hammarström[1]

[1]Division of Clinical Immunology, Department of Laboratory Medicine, Karolinska University Hospital Huddinge, Stockholm, Sweden; [2]Department of Immunopathology, SA Pathology, Women's and Children's Hospital Campus, and Robinson Research Institute and Discipline of Paediatrics, School of Medicine, University of Adelaide, North Adelaide, South Australia

Background. Single-nucleotide polymorphisms (SNPs) in the fucosyltransferase genes *FUT2* and *FUT3* have been associated with susceptibility to various infectious and inflammatory disorders. *FUT* variations influence the expression of human histo-blood group antigens (HBGAs) (H-type 1 and Lewis), which are highly expressed in the gut and play an important role in microbial attachment, metabolism, colonization, and shaping of the microbiome. In particular, *FUT* polymorphisms confer susceptibility to specific rotavirus and norovirus genotypes, which has important global health implications.

Methods. We designed a genotyping method using a nested polymerase chain reaction approach to determine the frequency of SNPs in *FUT2* and *FUT3*, thereby inferring the prevalence of Lewis^b-positive, Lewis^b-negative, secretor, and nonsecretor phenotypes in 520 Swedish newborns.

Results. There was an increased frequency of homozygotes for the minor allele for 1 SNP in *FUT2* and 4 SNPs in *FUT3*. Overall, 37.3% of newborns were found to have Lewis b negative phenotypes (Le (a^+b^-) or Le (a^-b^-). Using our new, sensitive genotyping method, we were able to genetically define the Le (a^-b^-) individuals based on their secretor status and found that the frequency of Lewis b negative newborns in our cohort was 28%.

Conclusions. Given the high frequency of fucosyltransferase polymorphisms observed in our newborn cohort and the implications for disease susceptibility, *FUT* genotyping might play a future role in personalized health care, including recommendations for disease screening, therapy, and vaccination.

Keywords. fucosyltransferase; *FUT2*; *FUT3*; Lewis^b; secretor; nonsecretor; rotavirus; norovirus.

The fucosyltransferase 2 (*FUT2*) and fucosyltransferase 3 (*FUT3*) genes give rise to H-type 1 and Lewis human histo-blood group antigens (HBGAs). Single-nucleotide polymorphisms (SNPs) located in these genes are associated with susceptibility or resistance to various infectious and inflammatory diseases and play a role in shaping the microbiome as a result of the influence of HBGAs on colonization patterns of the commensal intestinal flora. These antigens are highly expressed in the gut mucosa and secretions and are implicated in susceptibility to a range of microorganisms and other environmental stimuli [1]. HBGAs are receptors for various pathogens, including rotavirus, norovirus, *Helicobacter pylori*, and *Campylobacter jejuni*. The pattern of antigen expression by each individual, therefore, determines his or her susceptibility to infection [2–5]. In the case of *Escherichia coli*, HBGAs have been shown to contribute to microbial metabolism by providing a carbon source [6] and also to provide nutrition for other bacteria, including commensal flora [1]. As a result of these mechanisms, *FUT* polymorphisms play an important role in infectivity by pathogenic microorganisms, intestinal colonization with commensal flora, and shaping of the microbiome [7], and there are implications for host defense, intestinal homeostasis, and disease susceptibility.

The secretor (*FUT2*) gene is located on chromosome 19q13.3 and encodes the enzyme α-1,2-fucosyltransferase, which converts the type 1 chain precursor to H-type 1 antigen. The Lewis (*FUT3*) gene is located on chromosome 19p13.3 and encodes α-1,3-fucosyltransferase, which converts the H-type 1 antigen to Lewis^b and the type 1 chain precursor to Lewis^a [8, 9] (Figure 1). The presence of specific alleles in *FUT2* and *FUT3* result in differential gene expression, protein production, and enzyme activity [10, 11]. The prevalence of each haplotype and ensuing Lewis^b and secretor phenotype markedly differs between populations (Table 1) [12, 13]. A previous study in a Swedish population (of 207 healthy individuals) found 55% of the participants to

Received 1 April 2018; editorial decision 7 August 2018; accepted 26 October 2018; published online XXXX XX, XXXX.

Correspondence: J. Varadé, Division of Clinical Immunology, Department of Laboratory Medicine, Alfred Nobels Allé 8, Huddinge F79 14186 Stockholm, Sweden (jezabel.varade@ki.se).

Journal of the Pediatric Infectious Diseases Society XXXX;XX(XX):1–12

DOI: 10.1093/jpids/piy085

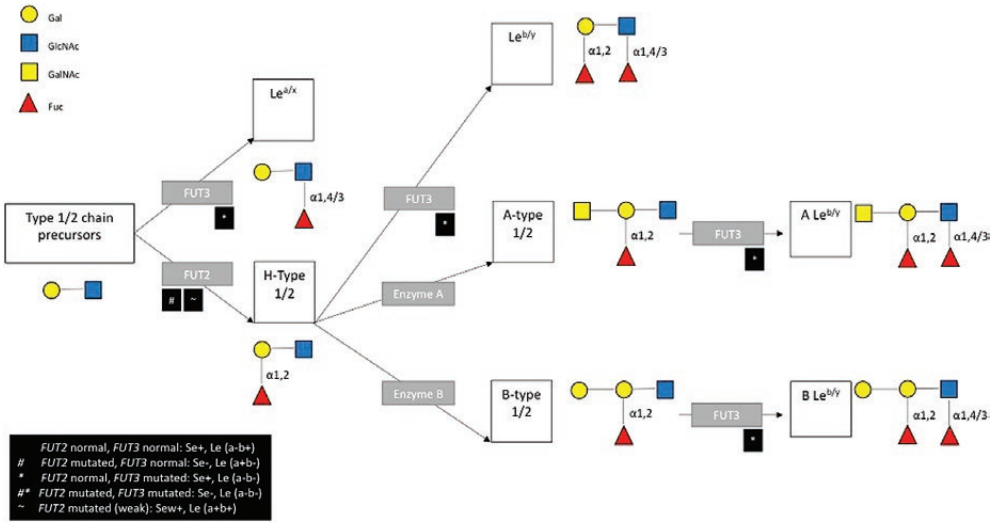


Figure 1. Histo-blood group antigen biosynthetic pathways from Type 1 and Type 2 precursors. *FUT2* generally encodes for Lewis and blood group antigen expression on Type 1 glycans, and *FUT1* generally encodes for Lewis expression on Type 2 glycans. Antigens are given in white boxes, key genes encoding enzymes are given in grey boxes. Effects of *FUT2* and *FUT3* mutations on enzyme expression and ensuing secretor, Lewis a and b phenotypes are given in black boxes. *FUT2* = α(1,2)fucosyltransferase, *FUT3* = α (1,3-4)fucosyltransferase 3, Enzyme A = Nacetylgalactosaminetransferase, Enzyme B = α-galactosyltransferase, Gal = D-galactose, GlcNAc = N-acetylglucosamine, GalNAc = N-acetylgalactosamine, Fuc = L-fucose.

be Lewis^b positive with a secretor phenotype [Se^+, $Le(a^-b^+)$], 31% were Lewis^b-negative nonsecretors [Se^-, $Le(a^+b^-)$], 11% were secretors with a Lewis-null phenotype [Se^+, $Le(a^-b^-)$], and 3% were nonsecretors and Lewis null [Se^-, $Le(a^-b^-)$] [14].

It was noted recently that H-type 1 and Lewis HBGAs are putative receptors for norovirus and rotavirus VP8* and thus play a role in viral attachment and entry into enterocytes [2, 15, 16]. Interindividual genetic variations (SNPs) in HBGAs have been found to confer either susceptibility or resistance to infection with specific norovirus and rotavirus genotypes [17]. Rotavirus infection is an important global health issue; it is a major cause of infectious gastroenteritis worldwide and accounts for approximately 215 000 child deaths annually, predominantly in developing countries [18]. Rotavirus is a nonenveloped double-stranded RNA virus that belongs to the *Reoviridae* family. Rotavirus strains P[4] and P[8] bind to Lewis^b and H-type 1 HBGAs, and strain P[6] binds to the H-type 1 HBGA alone. Hence, individuals who have a secretor phenotype (ie, express Lewis^b antigen) are more prone to rotavirus P[8] infection [11, 17], whereas nonsecretors have an intrinsic resistance to infection by these strains [11]. This finding is supported also by the observation that secretors produce higher levels of anti-rotavirus antibodies than nonsecretors [14]. Two safe and efficacious live attenuated rotavirus vaccines are available. Either monovalent Rotarix (GSK Biologicals, Brentford, United Kingdom) or multivalent RotaTeq (Merck & Co, New York, NY) is included in routine immunization schedules in many countries. However, it is not yet included in the Swedish childhood immunization program.

Table 1. Reported Prevalence of Secretor/Lewis^b Status in Different Populations[a]

	Population Prevalence (%)			
Status	Caucasian	African	Japanese	Chinese
Se^+, $Le(a^-b^+)$, secretor phenotype, Lewis^b positive	72	55	73	62
Se^-, $Le(a^+b^-)$, nonsecretor phenotype, Lewis^b negative	22	20	0.2	0
Se^+ or Se^-, $Le(a^-b^-)$, any secretor phenotype, Lewis-null phenotype	6	25	10	11
Sew^+, $Le(a^+b^+)$ (rare), Lewis^b-positive "weak" secretor	Rare	Rare	16.8	27

[a]Adapted from Reid et al [12] and Daniels and Bromilow [13].

Norovirus, an RNA virus in the family *Caliciviridae*, accounts for approximately 20% of all cases of acute gastroenteritis globally and represents an important public health issue because of its high rate of transmissibility [19]. Six genogroups of norovirus exist, and groups GI and GII account for the majority of infections [20]. A recent meta-analysis revealed that secretors were 4.2 times more likely to be infected with norovirus than were nonsecretors and had a 9.9 times greater risk of GII.4 genotype infection [20]. Secretors were found to have a 26.6 times greater risk of rotavirus infection than were nonsecretors [20]; 1 included study revealed that nonsecretor status was protective against severe rotavirus infection [21]. The authors of this study further highlighted population-specific differences in the frequency of *FUT2* polymorphisms, showing that the prevalence of nonsecretors was significantly lower in Hispanic children [21].

Given the implications of fucosyltransferase gene polymorphisms and ensuing Lewis^b and secretor phenotypes in determining disease susceptibility, we genotyped a cohort of Swedish

neonates to determine the frequencies of 4 SNPs in *FUT3* and 2 in *FUT2* and the prevalence of secretors, nonsecretors, Lewis^b-negative, and Lewis^b-positive neonates.

MATERIALS AND METHODS

Sample Recruitment

This study was carried out in accordance with the standing regional ethical committee and Karolinska Institutet policies, which permit the use of anonymized biological samples for research purposes. As part of the routine neonatal screening program, a Guthrie card specimen is collected from each Swedish newborn; blood from a heel punch is blotted onto filter paper and tested for a range of diseases in the first few days of life. A punch measuring 3.2 mm in diameter was taken from a dried blood spot from each of 520 anonymized newborn Guthrie cards from the Centre for Inherited Metabolic Diseases (Karolinska University Hospital Solna, Stockholm, Sweden).

Selection of *FUT2* and *FUT3* SNPs for Analysis

Two SNPs in *FUT2* (rs601338 and rs602662) and 4 in *FUT3* (rs778986, rs28362459, rs3894326, and rs3745635) were selected for analysis on the basis of previous publications that suggested that polymorphisms at these sites, either alone or in combination with other polymorphisms, confer abnormal fucosyltransferase enzyme activity and therefore are associated with secretor or nonsecretor Lewis^b-negative status (Supplementary Table 1).

In silico Analysis

Because *FUT2* and *FUT3* are partially duplicated in the genome, particularly in the genes *FUT1,* and *FUT5* and/or *FUT6* respectively, we assessed the specificity of the primers used in different publications for genotyping the SNPs rs601338 and rs602662 in *FUT2* and rs778986, rs28362459, rs3894326, and rs3745635 in *FUT3.* To establish whether the different primers described in the publications identified in the systematic review were specific, we checked the annealing region for each pair of primers using the Ensembl BLAST tool (see http://www.ensembl.org/index.html, last accessed: July 19, 2017). Primer pairs that annealed in more than 1 chromosomal location and produced an amplicon of less than 100 base pairs were classified as nonspecific.

Genotyping for SNPs in the *FUT2* and *FUT3* Genes

Genomic DNA was extracted from the neonatal dried blood spots using a DNA-extraction-kit method (Qiagen, Dusseldorf, Germany) according to manufacturer instructions. Genomic DNA was preamplified by polymerase chain reaction (PCR) using primers specific for the *FUT2* and *FUT3* gene regions containing SNPs of interest (Supplementary Table 2). Thirty nanograms of genomic DNA, 10 μL of 1× GoTaq colorless buffer (Promega, Madison, Wisconsin), 3 μL of 10 mM deoxyribonucleotide triphosphate (dNTP) (Invitrogen, Carlsbad, California), 3 μg of 1.5 mM/μL $MgCl_2$ (Promega), 2 μL of each forward and reverse amplification primer at 10 nM (Eurofins Scientific, Brussels, Belgium), 0.25 μL of GoTaq DNA polymerase (Promega), and 26.75 μL of distilled water were combined to result in a total reaction volume of 50 μL. Thermal cycler conditions were as follows: initial denaturation at 95°C for 2 minutes, followed by 30 cycles of denaturalization at 95°C for 30 seconds, annealing at 68°C (*FUT2*) or 60°C (*FUT3*) for 30 seconds, and extension at 72°C for 1 minute and then final extension at 72°C for 10 minutes. PCR products were visualized in 1% agarose gel. TaqMan chemistry (Life Technologies, Carlsbad, California) was used to genotype 2 SNPs in *FUT2* (rs601338 [C_2405292_10] and rs602662 [C_2405293_10]) and 2 SNPs in *FUT3* (rs3894326 [C_801690_10] and rs778986 [C_11458475_20]). Nontemplate negative controls and samples confirmed (by Sanger sequencing) to have mutant alleles were used as positive controls in the assays. Four microliters of amplified PCR product diluted 1:200 were used in the TaqMan reaction according to manufacturer conditions in a final volume of 20 μL (Life Technologies) and analyzed using a real-time PCR system under conditions recommended by the manufacturer (Applied Biosystems, Foster City, California). The *FUT3* SNPs rs3745635 and rs28362459 were genotyped using 4 μL of amplified PCR product diluted 1:80 as a template for Sanger sequencing.

Inferring Secretor and Lewis^b Status

Neonates found to carry the ancestral (wild-type) genotype at all evaluated *FUT2* and *FUT3* SNP sites were considered to have normal *FUT2* and *FUT3* expression and were classified as Lewis^b-positive secretors [Se^+, $Le(a^-b^+)$]. Neonates who were homozygous for the minor allele in *FUT2* at rs601338 and/or rs602662 with a wild-type *FUT3* genotype were classified as Lewis^b-negative nonsecretors [Se^-, $Le(a^+b^-)$]. Neonates homozygous for the minor allele in *FUT3* at rs778986, rs28362459, rs3894326, and/or rs3745635 with a wild-type *FUT2* genotype were considered to have a secretor Lewis-null phenotype [Se^+, $Le(a^-b^-)$], and those who were homozygous for the minor allele at 1 or more sites in *FUT2* and *FUT3* were considered to have a nonsecretor Lewis-null phenotype [Se^-, $Le(a^-b^-)$].

Statistical Analysis

The frequency of each of the 6 SNPs analyzed in our study population was determined and compared with data obtained from the 1000 Genomes Project European Caucasian (EUR) population (see http://www.internationalgenome.org, last accessed: July 3, 2017). As previously described, the frequencies of the secretor, nonsecretor, Lewis^b-negative, and Lewis-null phenotypes were determined on the basis of analysis of the pattern of SNPs present in each neonate. Statistical analyses were performed using SPSS Statistics 23 (IBM, Armonk, New York).

Systematic Review and Meta-analysis

To compare our genotyping results with those in other published cohorts in different populations, we conducted a systematic review and meta-analysis using PubMed (see http://www.ncbi.nlm.nih.gov/pubmed, last accessed: July 3, 2017), Medline Ovid (see http://www.ovid.com, last accessed: July 3, 2017) and the Cochrane Library (see http://www.cochranelibrary.com, last accessed: July 3, 2017) databases by applying the Medical Subject Heading terms "*FUT2*," "*FUT3*," "single nucleotide polymorphism," "secretor," "nonsecretor," "Lewis^b," and "Lewis^b negative." We also evaluated minor genotype frequencies for each site in the published studies and the 1000 Genomes Project for selected populations. Review Manager 5.0 (2008 Cochrane Collaboration, Oxford, United Kingdom) was used to carry out the statistical analysis. The stages of the systematic review and meta-analysis, including applied inclusion and exclusion criteria are shown in Figure 2.

RESULTS

Systematic Review and Meta-analysis

Of the 126 potentially eligible studies identified after initial exclusion of duplicates, errata, and meeting abstract communications, 96 were evaluated in more detail, and 17 were included

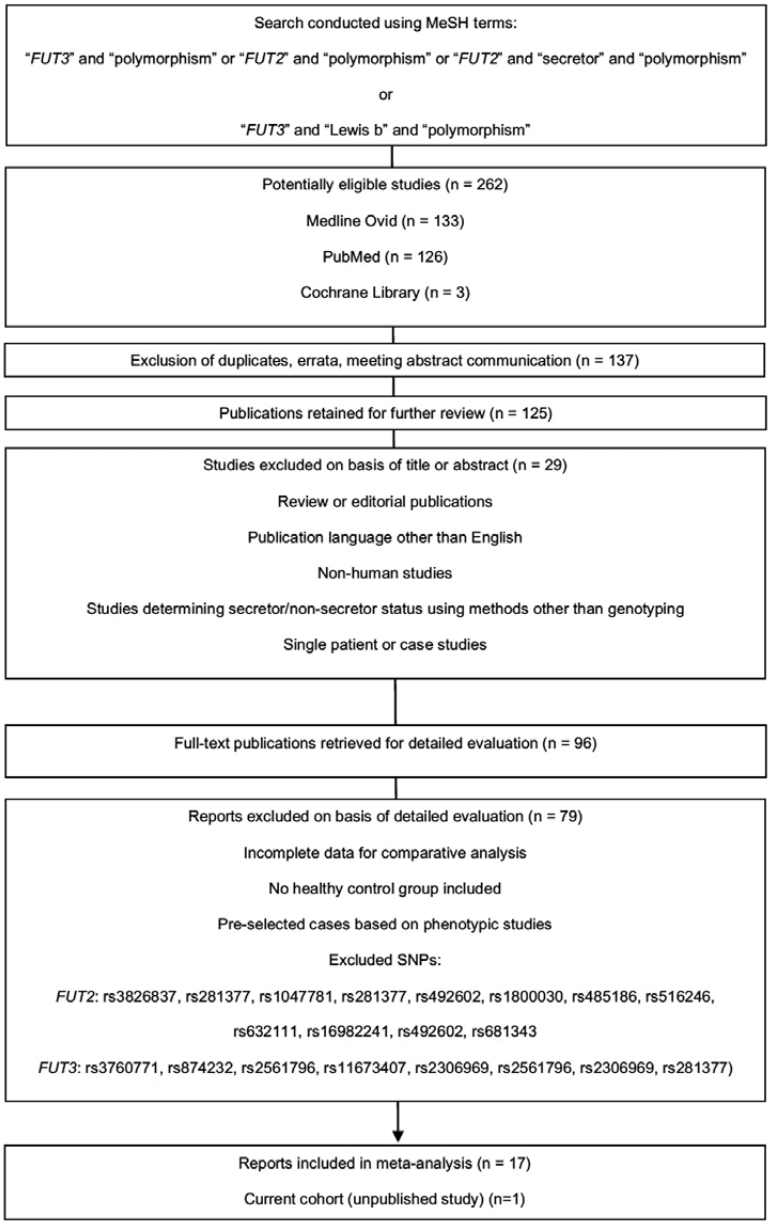
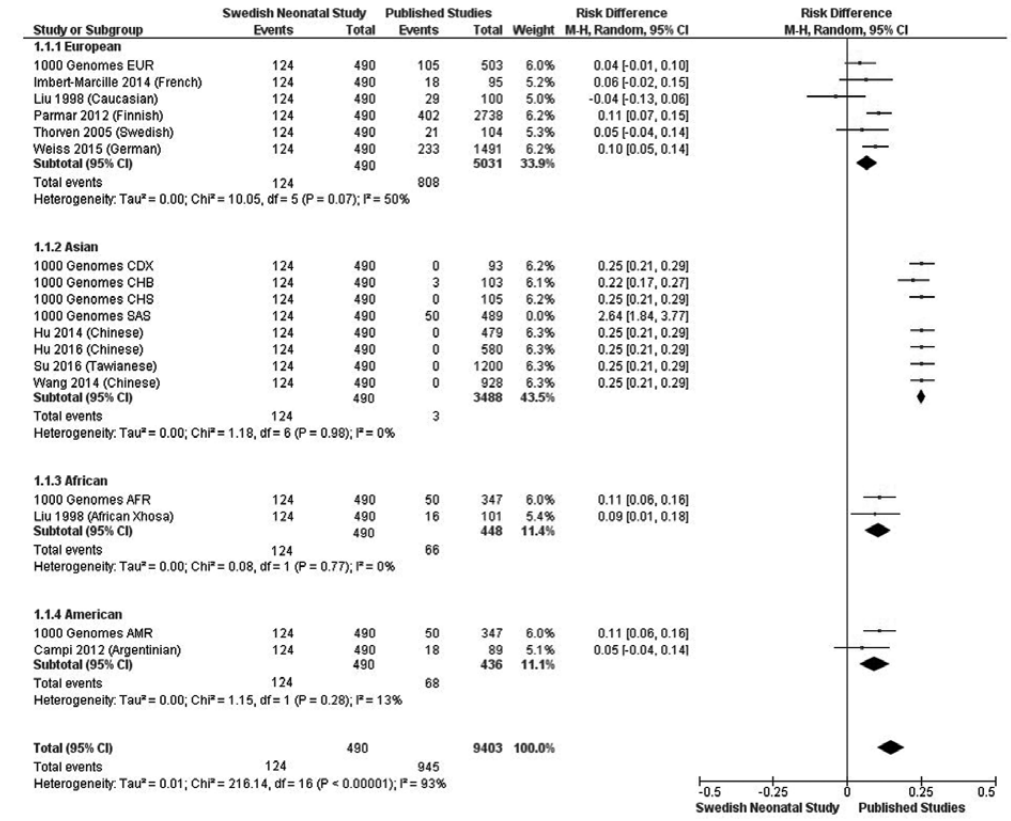


Figure 2. Flow diagram of systematic review and meta-analysis. Abbreviation: MeSH, Medical Subject Heading.

A **rs601338**



rs602662

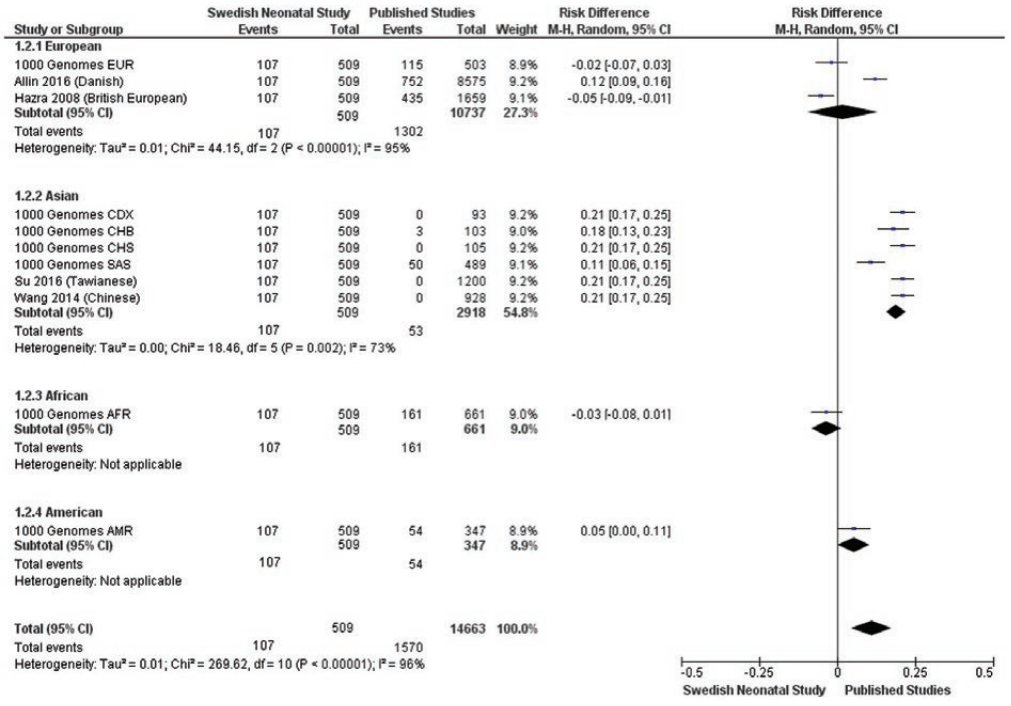
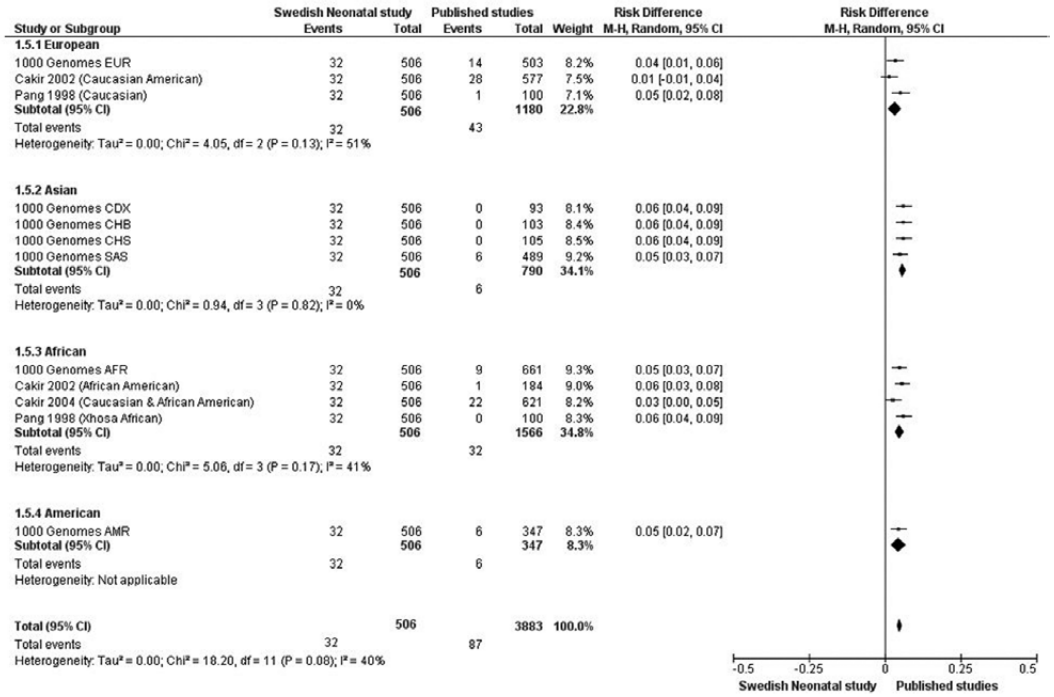


Figure 3. Forrest plots, minor genotype frequency for evaluated *FUT2* SNPs (A) and *FUT3* SNPs (B) in published studies, 1000 Genomes Project for selected populations and the current study. Abbreviation: AFR, African; AMR, American; CDX, Chinese Dai in Xishuangbanna China; CHB, Han Chinese in Beijing, China; CHS, Southern Han Chinese, China; CLM, Colombian in Medellin, Colombia; GBR, British in England and Scotland; EUR, European; FIN, Finnish in Finland; SAS, South Asian.

B **rs778986**



rs3894326

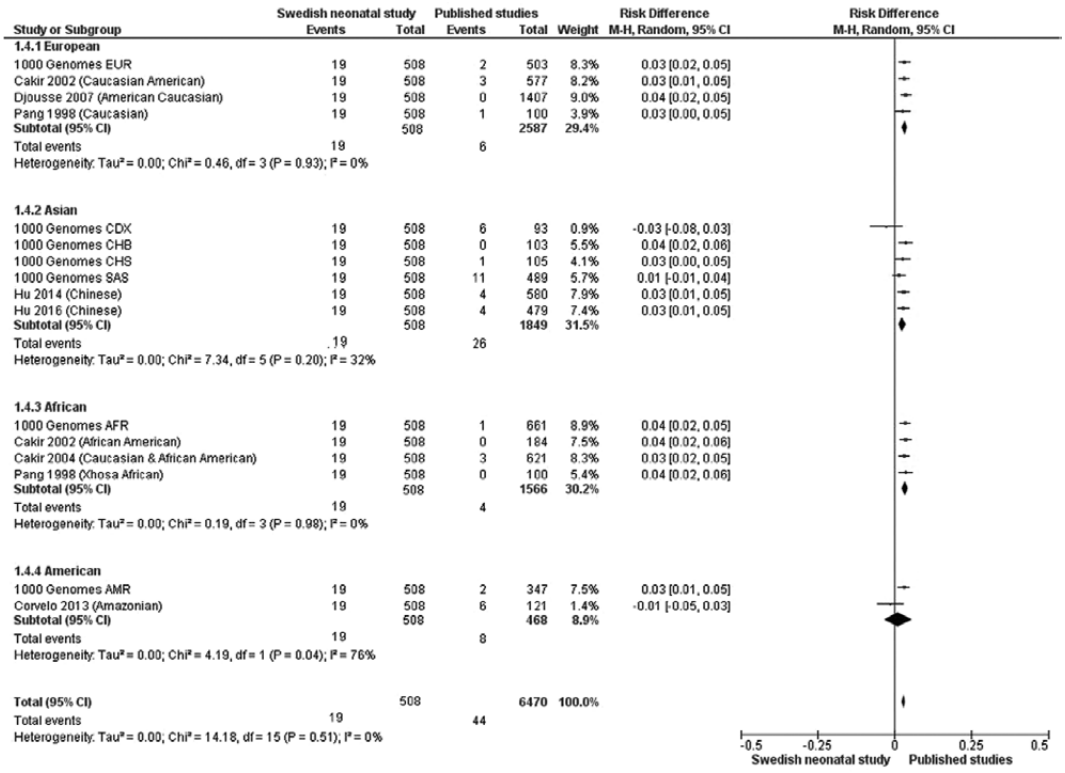
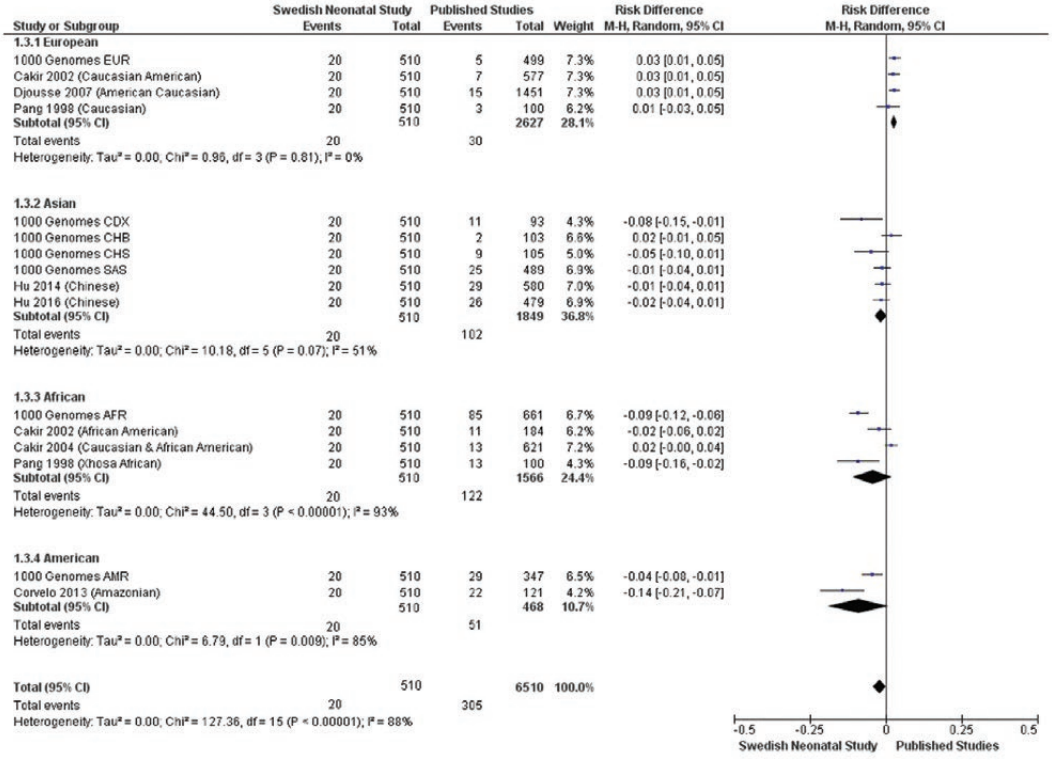


Figure 3. Continued.

rs28362459



rs3745635

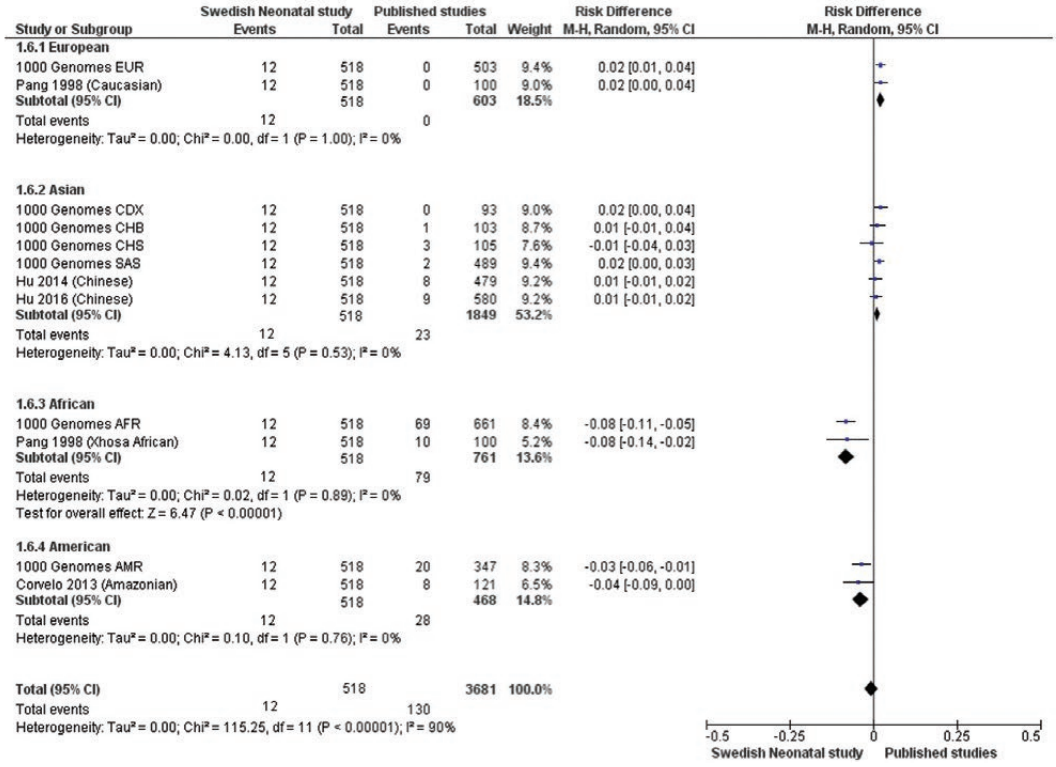


Figure 3. Continued.

Table 2. Genotype and Minor Allele Frequencies of Evaluated SNPs in *FUT2* and *FUT3*

SNP	Gene	Genotype Allele	Swedish Neonate Cohort (n [%])	1000 Genomes CEU Cohort (n [%])	*P*[a,b] (Homozygotes for Minor Allele)	OR (95% CI)[b]
rs601338	*FUT2*	AA	124 (25)	105 (21)	.097	1.28 (0.96–1.73)
		AG	161 (33)	234 (47)		
		GG	205 (42)	164 (33)		
rs602662	*FUT2*	AA	107 (21)	115 (23)	.479	0.90 (0.67–1.21)
		AG	228 (45)	241 (48)		
		GG	174 (34)	147 (29)		
rs778986	*FUT3*	TT	32 (6)	14 (3)	**.007**	**2.36 (1.24–4.48)**
		CT	132 (26)	153 (30)		
		CC	342 (68)	336 (67)		
rs3894326	*FUT3*	TT	19 (4)	2 (0.4)	**<.001**	**0.006 (0.004–0.01)**
		AT	99 (19)	69 (14)		
		AA	390 (77)	432 (86)		
rs28362459	*FUT3*	GG	20 (4)	5 (1)	**.003**	**4.07 (1.51–10.92)**
		GT	72 (14)	89 (18)		
		TT	418 (82)	409 (81)		
rs3745635	*FUT3*	AA	12 (2)	0 (0)	**.001**	**NA**
		AG	26 (5)	16 (3)		
		GG	480 (93)	487 (97)		

Abbreviations: CEU, Northern Europeans from Utah; CI, confidence interval; OR, odds ratio; SNP, single-nucleotide polymorphism.
[a]χ^2 test.
[b]Bold values indicates statistically significant results.

in the meta-analysis (Figure 2; Supplementary Table 3). The frequencies of the minor allele genotype for homozygotes for each evaluated SNP in our cohort were compared with those in other published studies and the 1000 Genomes Project for different populations (Figure 3). As expected, differences were noted in minor allele genotype frequencies in different populations.

SNP Genotyping Results

Statistically significant differences between the frequencies of the minor allele genotype for homozygotes for rs778986*T ($P = .007$), rs3894326*A ($P \leq 0.001$), rs28362459*G ($P = .003$), and rs3745635*A ($P = .001$) in *FUT3* in our cohort and those in the 1000 Genomes Project cohort were found (Table 2). We found no statistically significant difference in the frequencies of the minor allele genotype for homozygotes for the *FUT2* SNPs rs601338*A or rs602662*A. All except 1 SNP (rs602662) had a departure from Hardy–Weinberg equilibrium. We successfully genotyped 490 infants for rs601338, 509 for rs602662, 506 for rs778986, 508 for rs3894326, 510 for rs28362459, and 518 for rs3745635 (overall genotyping success rate for SNPs in this study, >95%).

Identification of a New SNP in FUT3

We identified 1 new SNP (G/T) at position 5844777 in the *FUT3* gene, 4 base pairs upstream of rs28362459 in 3 samples (National Center for Biotechnology Information submitted SNP number (ss) = 2137543878, rs1391064014). This SNP results in a synonymous change (Leu > Leu). One heterozygous (GT) and 2 homozygous (GG) neonates were identified (minor allele frequency of 0.01).

Inferred Prevalence of Secretor, Nonsecretor, Lewis[b]-Negative, and Lewis-Null Phenotypes

The most prevalent minor alleles in our cohort were rs601338*A in *FUT2* (124 infants [25%]) and rs778986*T in *FUT3* (32 infants [6%]). To calculate the prevalence of each inferred phenotype correlating with the *FUT2*/*FUT3* haplotypes in the Swedish population, we included all neonates genotyped for all 6 SNPs in this study, and 457 neonates were considered. In our cohort, 62.7% of the neonates were classified as Lewis[b] positive with a secretor phenotype [Se^+, $Le(a^-b^+)$], 23.5% were Lewis[b]-negative nonsecretors [Se^-, $Le(a^+b^-)$], 9.6% were considered to have a secretor Lewis-null phenotype [Se^+, $Le(a^-b^-)$], and 4.2% had a nonsecretor Lewis-null phenotype [Se^-, $Le(a^-b^-)$] (Table 3). In total, on the basis of SNP genotyping, 37.3% of the Swedish newborns were found to have Lewis b negative

Table 3. Prevalence of Inferred Secretor/Lewis[b] Phenotypes in a Swedish Neonatal Cohort

Secretor/Lewis[b] Phenotype	Prevalence (n [%])
Se^+, $Le(a^-b^+)$, secretor Lewis[b]-positive phenotype, normal *FUT2* expression, normal *FUT3* expression	286 (62.7)
Se^-, $Le(a^+b^-)$, nonsecretor Lewis[b]-negative phenotype, *FUT2* mutation, normal *FUT3* expression	107 (23.5)
Se^+, $Le(a^-b^-)$, secretor Lewis-null phenotype, normal *FUT2* expression, *FUT3* mutation	44 (9.6)
Se^-, $Le(a^-b^-)$, nonsecretor Lewis-null phenotype, *FUT2* mutation, *FUT3* mutation	19 (4.2)
Sew, $Le(a^+b^+)$ (rare), "weak" secretor Lewis[b]-positive phenotype, *FUT2* mutation (could not be assessed in this study)	NA

Abbreviation: NA, not available.

phenotypes (Le (a^+b^-) or Le (a^-b^-). However, using our new sensitive genotyping method, we were able to genetically define the Le(a^-b^-) individuals based on their secretor status. Overall, the frequency of Lewis b negative newborns was 28%, in keeping with the expected frequency in Caucasian populations [12, 13].

DISCUSSION

We noted statistically significant higher-than-expected frequencies of the minor allele genotype for homozygotes in all 4 SNPs in *FUT3* (rs778986*T, rs3894326*A, rs28362459*G, and rs3745635*A). The frequencies of the *FUT2* SNPs rs601338*A and rs602662*A were not significantly different than those in the 1000 Genomes Project cohort. We found an ensuing higher prevalence of secretors and Lewis^b-negative neonates in our cohort. Along with differences in methodology and genotyping techniques by which SNPs were identified in other studies and the variability of SNPs selected for analysis, other possible explanations for our findings include population heterogeneity and the presence of "weak" secretors (*Sew*) or compensatory transferases in our cohort. Weak-secretor phenotypes have been described for various populations and are associated with homozygosity for the minor allele genotype in *FUT2* SNPs A385T (rs1047781) in East Asian populations [22] and G739A (rs602662) and T839C in Portuguese populations [23].

FUT2 and *FUT3* gene segments are partially duplicated in the genome, particularly in *FUT1* and *FUT5* and/or *FUT6*. Hence, we designed a nested-PCR approach to ensure binding specificity of our secondary primers and probes to facilitate acquisition of the most accurate genotyping data. Depending on the genotyping technique used, the replication of *FUT2* and *FUT3* gene segments elsewhere in the genome indicates that there is a risk of nonspecific binding to irrelevant genomic segments and inaccurate genotyping results. However, the reliability of genotyping is improved markedly if a preamplification step is applied to genomic DNA using highly specific primers to isolate the areas of interest in *FUT2* and *FUT3*.

In addition to methodologic differences, it is likely that the number and combination of SNPs analyzed will affect the observed frequencies of various secretor and Lewis^b phenotypes in a population. Many *FUT2* and *FUT3* polymorphisms have been identified, each with variable frequencies in different populations, and evidence of functional effects on enzyme function for many, but not all, SNPs has been documented. Comparison between studies is difficult, given the heterogeneity in the assignment of Lewis^b and secretor statuses based on genotyping alone and variable combinations of evaluated SNPs. It is possible that the true prevalence is even higher because of the presence of additional as-yet-unidentified SNPs with functional effects. For example, we identified a new SNP in *FUT3* that might give rise to altered Lewis antigen expression, although the functional consequence of this SNP requires further exploration. The influences of heterozygous and compound heterozygous genotypes on enzyme activity have also been described; however, in the majority of studies, only homozygosity for the minor allele is considered when assigning Lewis^b-negative status. Hence, the lack of uniformity in SNPs selected for evaluation, and consideration of homozygous and heterozygous genotypes might explain discrepancies in the reported frequencies between the studies. Therefore, the true frequency of Lewis^b-negative individuals might be underestimated. Furthermore, the effect of copy-number variations, deletions, and fusions (particularly in *FUT2*) in some populations have been described, but they require further investigation [24, 25].

Population heterogeneity also might contribute to the higher minor allele genotype frequencies in the SNPs analyzed in our cohort. It is likely that a percentage of our cohort were of non-Caucasian ethnicity, which explains the deviation from Hardy–Weinberg equilibrium observed in all but 1 evaluated SNP. A small percentage of individuals have a *FUT2* mutation with a *Le*(a^+b^+) phenotype and are weak secretors, but it is a rare phenotype in Caucasians. However, the prevalence is higher in other populations (Table 1) [12, 13, 26] and is indistinguishable from a nonsecretor phenotype on the basis of genotyping methods alone. As such, a small number of neonates in our cohort might have been weak secretors. Furthermore, there might be a role for other compensatory transferases in those with *FUT2* polymorphisms such that they were phenotypically and functionally secretors, which could have inflated our nonsecretor frequency artificially.

In some studies, secretor and Lewis antigen status is classified phenotypically using antigen expression assays alone, without genotyping. It should be noted that the expression of HBGAs can change in people with an altered physiological state such as pregnancy and in those with malignancy [13, 27]. Although Lewis antigens are reported to be fully developed and detectable in saliva at birth, the presence of various red cell antigens is variable in the first weeks of life and does not reach full maturity until approximately 2 years of age [27, 28]. As such, in early childhood, genotyping-based assignment of secretor and Lewis^b status is more reliable than the detection of red cell antigens, but results are comparable to those in salivary antigen studies.

The Lewis antigen system is complex, and although our knowledge of the precise physiological role of these HBGAs remains incomplete, it has become apparent that fucosyltransferase polymorphisms and ensuing antigen expression are associated with predisposition to certain infections and diseases. As such, determining an individual's Lewis^b and secretor statuses is likely to have important implications for the provision of personalized medicine, including guidance of specific advice for prevention, screening, and management of disease. On a population level, understanding the prevalence of these polymorphisms has implications for public health and vaccination strategies.

FUT2 secretor status is associated with increased susceptibility to some genotypes of norovirus and rotavirus [11, 17], whereas nonsecretor status protects against infection [21]. Therefore, from a mechanistic perspective, vaccinating nonsecretor individuals who lack putative rotavirus P[8] receptors with a monovalent vaccine for this rotavirus genotype would be expected to elicit a poorer immune response and be less effective in these individuals. Vaccinating nonsecretors with a monovalent vaccine might be an explanation for the high rates of vaccine failure in countries such as Africa, where the frequency of Lewis^b-negative nonsecretor individuals is high. However, studies that compared the vaccine efficacy of monovalent and multivalent vaccines found that efficacies differed by only 1% in both moderate-to-high-income and developing-world settings [29, 30]. The immune response to rotavirus infection and mechanisms by which previous natural infection and immunization confer protection against subsequent infection are multifaceted, and the roles of neutralizing antibodies (both homotypic and heterotypic), interferon-mediated innate immune responses, and cell-mediated mechanisms have been investigated [31, 32]. The induction of serotype-specific neutralizing antibodies was thought to be critical for protection against rotavirus infection, resulting in the development of multivalent vaccines; however, the demonstrable efficacy of monovalent vaccines has challenged this premise [31]. A recent study found that heterotypic protective immunity to rotavirus is mediated by the production of heterotypic antibodies directed primarily toward the stalk (VP5*) of the viral attachment protein VP4, along with VP8* and surface glycoprotein VP7 [33]. Heterotypic antibody responses have been shown to confer protection against multiple rotavirus types despite monovalent vaccination [20, 34], although it remains unknown if the heterotypic antibody titers achieved for other viral strains are sufficient to confer adequate long-lasting protection.

Given the safety and efficacy of rotavirus vaccination and its demonstrated ability to reduce rotavirus-associated morbidity and death, its inclusion in routine immunization programs worldwide should be considered. In regions where rotavirus vaccination is not offered as a standard practice for all infants, evaluation of *FUT* polymorphisms and ensuing secretor and Lewis^b statuses could identify those infants who would derive the greatest benefit from rotavirus vaccination. From a mechanistic perspective, given the high prevalence of *FUT* polymorphisms in our population, a multivalent vaccine would be favorable.

Secretor status has been shown to play a role in the infectivity of other infectious diseases, including those caused by *C jejuni* [3], *H pylori* [4, 35], and human immunodeficiency virus (HIV) [36–38]. Blood group antigens facilitate *H pylori* binding to the gastric epithelium, and individuals who are secretors [4] and blood group O positive [35] are at an increased risk of infection and subsequent gastric ulceration. Nonsecretors seem to have a reduced risk of HIV infection, possibly as a result of the modification of mucosal surface carbohydrates [36], and reduced HIV-1 disease progression was found in some series [37, 38]. HBGAs provide a carbon source essential for the metabolism of bacteria such as *E coli*, thereby contributing to the virulence of these pathogens [6]. *FUT2* polymorphisms also have been associated with poor outcome and complications in premature infants, including Gram-negative sepsis and necrotizing enterocolitis [39], although these results were not reproduced in a subsequent cohort [40].

It has been found that *FUT* polymorphisms play a significant role in the shaping of the microbiome in terms of colonization with commensal microbiota. Intestinal dysbiosis is implicated in the pathogenesis of various autoimmune and inflammatory diseases [1]. Secretors likely have a better ability to stabilize their microbiome; secretion of HBGAs into body fluids confers an improved first line of defense against pathogens and other environmental elements, and beneficial bacteria thrive on glycosylation products [1]. *FUT2* polymorphisms have been implicated in some populations as a risk factor for Crohn disease [41–43], ulcerative colitis [43, 44], Behçet disease [45], celiac disease [43], and diabetes mellitus. In addition to being a risk factor for primary sclerosing cholangitis (PSC) [46], *FUT2* polymorphisms have been shown to influence biochemical parameters in patients with PSC, which affects interpretation of the carcinoembryonic antigen test that is used for malignancy screening [47]. These findings have important implications for surveillance and investigation in this patient group and might be applicable to screening for other gastrointestinal malignancies [47]. Serum lipase levels and the risk of chronic pancreatitis are elevated in nonsecretors [1]. Fucosyltransferase polymorphisms have been implicated also as risk factors for cardiovascular disease [10, 48] and various malignancies [49–51]. They have also been shown to influence serum metabolite pathways, including vitamin B_{12} levels [52, 53].

Given the implications of fucosyltransferase polymorphisms for disease susceptibility, screening of individuals might provide useful information to assist clinicians in providing personalized healthcare. Early identification of interindividual genetic differences in the newborn period would enable information to be readily available and actionable as needed, enabling accurate prediction of disease predisposition and facilitating appropriately informed decisions regarding screening, treatment, and preventative therapies such as vaccination. Furthermore, the role of HBGAs in disease pathogenesis should be considered in the development of novel treatment modalities and in vaccine development.

Supplementary Data

Supplementary materials are available at *Journal of the Pediatric Infectious Diseases Society* online.

Notes

Acknowledgments. J. R. K. is the recipient of the Mike and Carole Ralston Travelling Fellowship 2016 from the RCPA Foundation. We acknowledge Lennart Svensson, Thomas Boren, and Johan Nordgren for their critical review of the manuscript. We sincerely thank Ulrika von Döbeln for provision of the DBS specimens and review of the manuscript.

Financial support. This work was supported by grants from Vetenskapsrådet and the Stockholm County Council (ALF project) to L. H.

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Weiss FU, Schurmann C, Guenther A, et al. Fucosyltransferase 2 (FUT2) non-secretor status and blood group B are associated with elevated serum lipase activity in asymptomatic subjects, and an increased risk for chronic pancreatitis: a genetic association study. Gut **2015**; 64:646–56.
2. Hu L, Crawford SE, Czako R, et al. Cell attachment protein VP8* of a human rotavirus specifically interacts with A-type histo-blood group antigen. Nature **2012**; 485:256–9.
3. Ruiz-Palacios GM, Cervantes LE, Ramos P, et al. *Campylobacter jejuni* binds intestinal H(O) antigen (Fuc alpha 1, 2Gal beta 1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection. J Biol Chem **2003**; 278:14112–20.
4. Borén T, Falk P, Roth KA, et al. Attachment of *Helicobacter pylori* to human gastric epithelium mediated by blood group antigens. Science **1993**; 262:1892–5.
5. Marionneau S, Airaud F, Bovin NV, et al. Influence of the combined ABO, FUT2, and FUT3 polymorphism on susceptibility to Norwalk virus attachment. J Infect Dis **2005**; 192:1071–7.
6. Pacheco AR, Curtis MM, Ritchie JM, et al. Fucose sensing regulates bacterial intestinal colonization. Nature **2012**; 492:113–7.
7. Wacklin P, Tuimala J, Nikkilä J, et al. Faecal microbiota composition in adults is associated with the FUT2 gene determining the secretor status. PLoS One **2014**; 9:e94863.
8. Taube S, Jiang M, Wobus CE. Glycosphingolipids as receptors for non-enveloped viruses. Viruses **2010**; 2:1011–49.
9. Orntoft TF, Holmes EH, Johnson P, et al. Differential tissue expression of the Lewis blood group antigens: enzymatic, immunohistologic, and immunochemical evidence for Lewis a and b antigen expression in *Le*(*a-b-*) individuals. Blood **1991**; 77:1389–96.
10. Salomaa V, Pankow J, Heiss G, et al. Genetic background of Lewis negative blood group phenotype and its association with atherosclerotic disease in the NHLBI family heart study. J Intern Med **2000**; 247:689–98.
11. CI, CDX, CHB, CHS, SAS, AFR, -Marcille BM, Barbé L, Dupé M, et al. A *FUT2* gene common polymorphism determines resistance to rotavirus A of the P[8] genotype. J Infect Dis **2014**; 209:1227–30.
12. Reid C, Lomas-Francis C, Olsson M. Lewis blood group system. In: Reid M, Olsson LFM, ed. The Blood Group Antigen Factsbook. 3rd ed. Elsevier; **2012**.
13. Daniels G, Bromilow I. Essential Guide to Blood Groups. 3rd ed. John Wiley & Sons, Ltd; **2014**.
14. Günaydın G, Nordgren J, Sharma S, Hammarström L. Association of elevated rotavirus-specific antibody titers with HBGA secretor status in Swedish individuals: the *FUT2* gene as a putative susceptibility determinant for infection. Virus Res **2016**; 211:64–8.
15. Huang P, Xia M, Tan M, et al. Spike protein VP8* of human rotavirus recognizes histo-blood group antigens in a type-specific manner. J Virol **2012**; 86:4833–43.
16. Böhm R, Fleming FE, Maggioni A, et al. Revisiting the role of histo-blood group antigens in rotavirus host-cell invasion. Nat Commun **2015**; 6:5907.
17. Nordgren J, Sharma S, Bucardo F, et al. Both Lewis and secretor status mediate susceptibility to rotavirus infections in a rotavirus genotype-dependent manner. Clin Infect Dis **2014**; 59:1567–73.
18. Tate JE, Burton AH, Boschi-Pinto C, Parashar UD; World Health Organization–Coordinated Global Rotavirus Surveillance Network. Global, regional, and national estimates of rotavirus mortality in children <5 years of age, 2000–2013. Clin Infect Dis **2016**; 62(Suppl 2):S96–105.
19. Currier RL, Payne DC, Staat MA, et al. Innate susceptibility to norovirus infections influenced by *FUT2* genotype in a United States pediatric population. Clin Infect Dis **2015**; 60:1631–8.
20. Kambhampati A, Payne DC, Costantini V, Lopman BA. Host genetic susceptibility to enteric viruses: a systematic review and metaanalysis. Clin Infect Dis **2016**; 62:11–8.
21. Payne DC, Currier RL, Staat MA, et al. Epidemiologic association between *FUT2* secretor status and severe rotavirus gastroenteritis in children in the United States. JAMA Pediatr **2015**; 169:1040–5.
22. Silva LM, Carvalho AS, Guillon P, et al. Infection-associated *FUT2* (fucosyltransferase 2) genetic variation and impact on functionality assessed by in vivo studies. Glycoconj J **2010**; 27:61–8.
23. Serpa J, Mendes N, Reis CA, et al. Two new *FUT2* (fucosyltransferase 2 gene) missense polymorphisms, 739G–>A and 839T–>C, are partly responsible for non-secretor status in a Caucasian population from northern Portugal. Biochem J **2004**; 383:469–74.
24. Park KU, Song J, Han KS, Kim JQ. The fusion allele of the *FUT2* (secretor type alpha(1,2)-fucosyltransferase) gene at a high frequency and a new *se385* allele in a Korean population. Ann Hematol **2005**; 84:656–60.
25. Soejima M, Koda Y. TaqMan-based real-time polymerase chain reaction for detection of *FUT2* copy number variations: identification of novel Alu-mediated deletion. Transfusion **2011**; 51:762–9.
26. Henry SM, Benny AG, Woodfield DG. Investigation of Lewis phenotypes in Polynesians: evidence of a weak secretor phenotype. Vox Sang **1990**; 58:61–6.
27. Assali NS. Pathophysiology of Gestation. New York: Academic Press; **1972**.
28. Henry S, Oriol R, Samuelsson B. Lewis histo-blood group system and associated secretory phenotypes. Vox Sang **1995**; 69:166–82.
29. Shah MP, Tate JE, Mwenda JM, et al. Estimated reductions in hospitalizations and deaths from childhood diarrhea following implementation of rotavirus vaccination in Africa. Expert Rev Vaccines **2017**; 16:987–95.
30. Cortese MM, Immergluck LC, Held M, et al. Effectiveness of monovalent and pentavalent rotavirus vaccine. Pediatrics **2013**; 132:e25–33.
31. Ward RL, Clark HF, Offit PA. Influence of potential protective mechanisms on the development of live rotavirus vaccines. J Infect Dis **2010**; 202(Suppl):S72–9.
32. Angel J, Franco MA, Greenberg HB. Rotavirus immune responses and correlates of protection. Curr Opin Virol **2012**; 2:419–25.
33. Nair N, Feng N, Blum LK, et al. VP4- and VP7-specific antibodies mediate heterotypic immunity to rotavirus in humans. Sci Transl Med **2017**; 9:eaam5434.
34. Leshem E, Lopman B, Glass R, et al. Distribution of rotavirus strains and strain-specific effectiveness of the rotavirus vaccine after its introduction: a systematic review and meta-analysis. Lancet Infect Dis **2014**; 14:847–56.
35. Azevedo M, Eriksson S, Mendes N, et al. Infection by *Helicobacter pylori* expressing the BabA adhesin is influenced by the secretor phenotype. J Pathol **2008**; 215:308–16.
36. Ali S, Niang MA, N'doye I, et al. Secretor polymorphism and human immunodeficiency virus infection in Senegalese women. J Infect Dis **2000**; 181:737–9.
37. Bigham AW, Mackelprang RD, Celum C, et al. Variants in host viral replication cycle genes are associated with heterosexual HIV-1 acquisition in Africans. J Acquir Immune Defic Syndr **2014**; 66:127–34.
38. Kindberg E, Hejdeman B, Bratt G, et al. A nonsense mutation (428G–>A) in the fucosyltransferase *FUT2* gene affects the progression of HIV-1 infection. AIDS **2006**; 20:685–9.
39. Morrow AL, Meinzen-Derr J, Huang P, et al. Fucosyltransferase 2 non-secretor and low secretor status predicts severe outcomes in premature infants. J Pediatr **2011**; 158:745–51.
40. Demmert M, Schaper A, Pagel J, et al. *FUT 2* polymorphism and outcome in very-low-birth-weight infants. Pediatr Res **2015**; 77:586–90.
41. McGovern DP, Jones MR, Taylor KD, et al.; International IBD Genetics Consortium. Fucosyltransferase 2 (FUT2) non-secretor status is associated with Crohn's disease. Hum Mol Genet **2010**; 19:3468–76.
42. Hu DY, Shao XX, Xu CL, et al. Associations of *FUT2* and *FUT3* gene polymorphisms with Crohn's disease in Chinese patients. J Gastroenterol Hepatol **2014**; 29:1778–85.
43. Parmar AS, Alakulppi N, Paavola-Sakki P, et al. Association study of *FUT2* (rs601338) with celiac disease and inflammatory bowel disease in the Finnish population. Tissue Antigens **2012**; 80:488–93.
44. Aheman A, Luo HS, Gao F. Association of fucosyltransferase 2 gene variants with ulcerative colitis in Han and Uyghur patients in China. World J Gastroenterol **2012**; 18:4758–64.
45. Xavier JM, Shahram F, Sousa I, et al. *FUT2*: filling the gap between genes and environment in Behçet's disease? Ann Rheum Dis **2015**; 74:618–24.
46. Folseraas T, Melum E, Rausch P, et al. Extended analysis of a genome-wide association study in primary sclerosing cholangitis detects multiple novel risk loci. J Hepatol **2012**; 57:366–75.
47. Wannhoff A, Folseraas T, Brune M, et al. A common genetic variant of fucosyltransferase 2 correlates with serum carcinoembryonic antigen levels and affects cancer screening in patients with primary sclerosing cholangitis. United European Gastroenterol J **2016**; 4:84–91.

48. Cakir B, Heiss G, Pankow JS, et al. Association of the Lewis genotype with cardiovascular risk factors and subclinical carotid atherosclerosis: the Atherosclerosis Risk in Communities (ARIC) study. J Intern Med **2004**; 255:40–51.
49. Duell EJ, Bonet C, Muñoz X, et al. Variation at ABO histo-blood group and *FUT* loci and diffuse and intestinal gastric cancer risk in a European population. Int J Cancer **2015**; 136:880–93.
50. Teresa DB, Santos RA, Takahashi CS, et al. Polymorphisms of Lewis and secretor genes are related to breast cancer and metastasis in axillary lymph nodes. Tumour Biol **2010**; 31:401–9.
51. Padró M, Cobler L, Garrido M, de Bolós C. Down-regulation of *FUT3* and *FUT5* by shRNA alters Lewis antigens expression and reduces the adhesion capacities of gastric cancer cells. Biochim Biophys Acta **2011**; 1810:1141–9.
52. Zinck JW, de Groh M, MacFarlane AJ. Genetic modifiers of folate, vitamin B-12, and homocysteine status in a cross-sectional study of the Canadian population. Am J Clin Nutr **2015**; 101:1295–304.
53. Tanwar VS, Chand MP, Kumar J, et al. Common variant in *FUT2* gene is associated with levels of vitamin B(12) in Indian population. Gene **2013**; 515:224–8.

7.3 Supplementary Material

Table 4: Characteristics of *FUT2* and *FUT3* SNPs selected for analysis

Gene	rs candidate	Flanking sequence	Ancestral allele	EUR Allele frequency (count)	EUR Genotype frequency (count)	SIFT score	Polyphen score
FUT2	rs601338 (G428A)	CGCTTCACCGGCTACCCCTG CTCCT[A/G]GACCTTCTACCAC CACCTCCGCCAG	G	G: 0.559 (562) A: 0.441 (444) MAF 0.2	G|G 0.326 (164) A|A: 0.209 (105) A|G: 0.465 (234)	-	-
FUT2	rs602662 (G739A)	TCGGGAGAACATTGACACCTC CCAC[A/G]GTGATGTGGTGTT TGCTGGCGATGG	G	G: 0.532 (535) A: 0.468 (471) MAF 0.33	G|G: 0.292 (147) A|A: 0.229 (115) A|G: 0.479 (241)	0.07[#]	0.986[###]
FUT3	rs28362459 (T59G)	CCATGGCGCCGCTGTCTGGC CGCAC[T/G]GCTATTTCAGCT GCTGGTGGCTGTG	T	T: 0.902 (907) G: 0.098 (99) MAF 0.01	T|T: 0.813 (409) T|G: 0.177 (89) G|G: 0.010 (5)	0.00[###]	0.964[###]
FUT3	rs3894326 (A1067T)	TCCAGGTACCAGACGGTGCG CAGCA[A/T]AGCGGCTTGGTT CACCTGAGAGGCC	A	A: 0.927 (933) T: 0.073 (73) MAF 0.04	A|A: 0.859 (432) A|T: 0.137 (69) T|T: 0.004 (2)	0.00[###]	0.859[##]
FUT3	rs778986 (C314T)	GATATCCCAGTGGTGCACGAT GACC[C/T]TGTCTGCCTGTGG GTACACCTTGCG	C	C: 0.180 (181) T: 0.820 (825) MAF 0.03	C|C: 0.028 (14) T|T: 0.304 (153) T|T: 0.668 (336)	0.01[###]	0.000[#]
FUT3	rs3745635 (C508T)	CATGTCCTACCGCAGCGACT CCGACATCTTCACGCCCTAC [C/T]GCTGGCTGGAGCCGTGG TCC	C	C: 0.984 (990) T: 0.016 (16) MAF 0.15	C|C: 0.968 (487) C|T: 0.032 (16)	0.04[###]	0.866[##]

EUR = European population, 1000 Genomes Project. MAF = minor allele frequency. SIFT (Sorting Tolerant From Intolerant) prediction results: [#] tolerated, [###] deleterious. Polyphen prediction results: [#] benign, [##] possibly damaging, [###] probably damaging.

Table 5: *FUT2* and *FUT3* amplification primers

Gene / rs candidate	Forward Primer (5' – 3')	Reverse Primer (5' – 3')	Size (bp)	Reference
FUT2	TCAACATCAAAGGCACTGGGACC*	GGAGTGGACAGGGATTGCCG[#]	856	*Hu et al. [42], [#]Tanwar et al. [53]
FUT3	ACACCAGCTGTGCTGATTTTT	GACCTTGGCTGCTGGAGG	1499	Designed
rs3745635	TGGAAGCCCTGGACAGATAC	AACTGGAAGCCGGACTCAG	177	Designed
rs28362459	GGAGCTTTGGTAAGCAGGAG	GAAGAAACACACAGCCACCA	167	Designed

Table 6: References included in the meta-analysis

1. Weiss FU, Schurmann C, Guenther A, et al. Fucosyltransferase 2 (FUT2) non-secretor status and blood group B are associated with elevated serum lipase activity in asymptomatic subjects, and an increased risk for chronic pancreatitis: a genetic association study. Gut 2015; 64:646-56.
2. Imbert-Marcille BM, Barbe L, Dupe M, et al. A FUT2 gene common polymorphism determines resistance to rotavirus A of the P[8] genotype. J Infect Dis 2014; 209:1227-30.
3. Cakir B, Heiss G, Pankow JS, et al. Association of the Lewis genotype with cardiovascular risk factors and subclinical carotid atherosclerosis: the Atherosclerosis Risk in Communities (ARIC) study. J Intern Med 2004; 255:40-51.
4. Cakir B, Pankow JS, Salomaa V, et al. Distribution of Lewis (FUT3)genotype and allele: frequencies in a biethnic United States population. Ann Hematol 2002; 81:558-65.
5. Corvelo TC, de Loiola Rdo S, Aguiar DC, de Matos Gde C, de Brito DC. The Lewis histo-blood group system: molecular analysis of the 59T>G, 508G>A, and 1067T>A polymorphisms in an Amazonian population. PloS one 2013; 8:e69908.
6. Djousse L, Karamohamed S, Herbert AG, D'Agostino RB, Cupples LA, Ellison RC. Fucosyltransferase 3 polymorphism and atherothrombotic disease in the Framingham Offspring Study. American heart journal 2007; 153:636-9.
7. Campi C, Escovich L, Moreno A, et al. Expression of the gene encoding secretor type galactoside 2 alpha fucosyltransferase (FUT2) and ABH antigens in patients with oral lesions. Medicina oral, patologia oral y cirugia bucal 2012; 17:e63-8.
8. Liu Y, Koda Y, Soejima M, et al. Extensive polymorphism of the FUT2 gene in an African (Xhosa) population of South Africa. Human genetics 1998; 103:204-10.
9. Pang H, Liu Y, Koda Y, et al. Five novel missense mutations of the Lewis gene (FUT3) in African (Xhosa) and Caucasian populations in South Africa. Human genetics 1998; 102:675-80.
10. Parmar AS, Alakulppi N, Paavola-Sakki P, et al. Association study of FUT2 (rs601338) with celiac disease and inflammatory bowel disease in the Finnish population. Tissue Antigens 2012; 80:488-93.
11. Thorven M, Grahn A, Hedlund KO, et al. A homozygous nonsense mutation (428G-->A) in the human secretor (FUT2) gene provides resistance to symptomatic norovirus (GGII) infections. J Virol 2005; 79:15351-5.
12. Hazra A, Kraft P, Selhub J, et al. Common variants of FUT2 are associated with plasma vitamin B12 levels. Nat Genet 2008; 40:1160-2.
13. Su KJ, Ho CC, Lin CW, et al. Combinations of FUT2 gene polymorphisms and environmental factors are associated with oral cancer risk. Tumour Biol 2016; 37:6647-52.
14. Wang J, Zhao JY, Wang F, et al. A genetic variant in vitamin B12 metabolic genes that reduces the risk of congenital heart disease in Han Chinese populations. PloS one 2014; 9:e88332.
15. Allin KH, Friedrich N, Pietzner M, et al. Genetic determinants of serum vitamin B12 and their relation to body mass index. European Journal of Epidemiology 2017; 32:125-34.
16. Hu D, Zhang D, Zheng S, Guo M, Lin X, Jiang Y. Association of Ulcerative Colitis with FUT2 and FUT3 Polymorphisms in Patients from Southeast China. PloS one 2016; 11:e0146557.
17. Hu DY, Shao XX, Xu CL, et al. Associations of FUT2 and FUT3 gene polymorphisms with Crohn's disease in Chinese patients. J Gastroenterol Hepatol 2014; 29:1778-85.

CHAPTER 8: FUTURE DIRECTIONS: NEWBORN SCREENING FOR PRIMARY IMMUNODEFICIENCY DISEASES IN THE NEXT GENERATION

8.1 Introduction and Contextual Statement

We have seen a rapid evolution in newborn screening technologies, and the future holds promise to expand our current capabilities. Currently, we are able to screen for a selected number of diseases using the TREC/KREC assay. Other methodologies, including mass spectrometry, sequencing-based and protein-based assays have been evaluated to determine their role in expanding our current repertoire of screened diseases. However, it has become apparent that NGS may have a future role in simultaneously screening infants for a large number of heterogeneous diseases. This chapter examines the future of newborn screening for PID, including the application of NGS-based approaches and issues for consideration prior to adopting this approach.

8.2 Publication: Newborn Screening for Primary Immunodeficiency Diseases: The Past, the Present and the Future

This constitutes the final published paper included in this thesis. The following paper, entitled 'Newborn Screening for Primary Immunodeficiency Diseases: The past, the present and the future', by Jovanka King, Jonas Ludvigsson and Lennart Hammarström was published in the peer reviewed journal, *International Journal of Neonatal Screening*, in August 2017 (3(3),19, doi:10.3390/ijns3030019).

ARTICLE METRICS (AS AT 11 JANUARY 2019):

Journal Impact Factor: Not available

Citations: 10

Altmetrics score: 4

Statement of Authorship

Title of Paper	Newborn Screening for Primary Immunodeficiency Diseases: The Past, the Present and the Future
Publication Status	Published
Publication Details	King, J.; Ludvigsson, J.F.; Hammarström, L. Newborn Screening for Primary Immunodeficiency Diseases: The Past, the Present and the Future. *Int. J. Neonatal Screen.* 2017, *3*, 19.

Principal Author

Name of Principal Author (Candidate)	Dr Jovanka King		
Contribution to the Paper	Literature review, manuscript design, database establishment, data analysis, synthesis of tables & figures, writing of manuscript.		
Overall percentage (%)	90%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	11/4/18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Professor Lennart Hammarström		
Contribution to the Paper	Oversight of manuscript content & structure, provision of critical review of manuscript.		
Signature		Date	2018-04-11

Title of Paper	Newborn Screening for Primary Immunodeficiency Diseases: The Past, the Present and the Future
Publication Status	Published
Publication Details	King, J.; Ludvigsson, J.F.; Hammarström, L. Newborn Screening for Primary Immunodeficiency Diseases: The Past, the Present and the Future. *Int. J. Neonatal Screen.* 2017, *3*, 19.

Name of Co-Author	Professor Jonas Ludvigsson		
Contribution to the Paper	Provision of data, critical review of manuscript.		
Signature		Date	180412

International Journal of
Neonatal Screening

MDPI

Review

Newborn Screening for Primary Immunodeficiency Diseases: The Past, the Present and the Future

Jovanka King [1,2], Jonas F. Ludvigsson [3,4] and Lennart Hammarström [1,*]

1 Department of Clinical Immunology, Karolinska University Hospital Huddinge, SE-14186 Stockholm, Sweden; jovanka.king@ki.se
2 Department of Immunopathology, SA Pathology, Women's and Children's Hospital Campus, Robinson Research Institute and Discipline of Paediatrics, School of Medicine, University of Adelaide, North Adelaide 5006, Australia
3 Department of Medical Epidemiology and Biostatistics, Karolinska Institutet, SE-10435 Stockholm, Sweden; jonas.ludvigsson@ki.se
4 Department of Gastroenterology, Faculty of Medicine and Health, Örebro University, SE-70281 Örebro, Sweden
* Correspondence: lennart.hammarstrom@ki.se

Received: 18 July 2017; Accepted: 1 August 2017; Published: 3 August 2017

Abstract: Primary immunodeficiency diseases (PID) are a heterogeneous group of disorders caused by inborn errors of immunity, with affected children presenting with severe, recurrent or unusual infections. Over 300 distinct genetic molecular abnormalities resulting in PID have been identified, and this number continues to rise. Newborn screening for PID has been established in many countries, with the majority of centers using a PCR-based T cell receptor excision circle (TREC) assay to screen for severe combined immunodeficiency (SCID) and other forms of T cell lymphopenia. Multiplexed screening including quantitation of kappa-recombining exclusion circles (KREC) has also been described, offering advantages over TREC screening alone. Screening technologies are also expanding to include protein-based assays to identify complement deficiencies and granulocyte disorders. Given the rapid advances in genomic medicine, a potential future direction is the application of next-generation sequencing (NGS) technologies to screen infants for a panel of genetic mutations, which would enable identification of a wide range of diseases. However, several ethical and economic issues must be considered before moving towards this screening strategy.

Keywords: newborn screening; primary immunodeficiency diseases; TREC; KREC; next-generation sequencing

1. Introduction

Primary immunodeficiency diseases (PID) are a heterogeneous group of disorders which are genetically determined inborn errors of immunity. In excess of 300 distinct genetic molecular abnormalities resulting in PID have been identified, and this number continues to increase. Based on estimates from the human connectome, more than 1000 genes interact with known PID genes [1]. Children with PID present with severe, recurrent or unusual infections, and these diseases are associated with significant morbidity and mortality. Until recent years, there was no available testing modality to identify these children prior to the onset of symptoms, frequently resulting in delayed diagnosis and treatment, and a complicated clinical course. Currently available newborn screening technologies have enabled the early identification of severe forms of PID, manifested by T and B cell lymphopenia, which has been demonstrated to have a profound impact on patient outcomes [2]. PID were previously thought to represent rare disease entities, however through newborn screening programs, true disease incidence rates can be determined and have proven to be higher than expected.

Newborn screening for PID continues to be an evolving field, with the potential for future expansion to include screening for other forms of PID such as granulocyte disorders and complement deficiency. The future of newborn screening for the identification of PID and other inborn errors is likely to involve a change in screening strategy, where next-generation sequencing will have an increasingly prominent role, potentially even as up-front testing. Here, we review the past and present aspects of newborn screening for primary immunodeficiency diseases, and discuss potential future directions.

2. The Past: Identification of Severe Combined Immunodeficiency as a Priority for Newborn Screening

Since the initiation of population-based newborn screening using dried blood spots (DBS) in the 1960s using a method established by Guthrie and Susi [3], there have been significant advances in our ability to screen asymptomatic infants for severe, life-threatening diseases for which treatment is available, and where early diagnosis and treatment is essential for preventing serious sequelae. For inclusion in population-based screening programs, diseases must meet a series of criteria as described by Wilson and Jungner [4]. Severe combined immunodeficiency (SCID) is a life-threatening condition resulting from a profound lymphocyte deficiency. It manifests with significant infections and is uniformly fatal without treatment. SCID is curable with hematopoietic stem cell transplantation (HSCT), and it has been demonstrated that outcomes are markedly improved for infants who are diagnosed and undergo HSCT prior to the age of 3.5 months [2]. Diagnosis and treatment is frequently delayed, and realistically, achieving this target is only possible if affected newborns are identified by screening prior to the onset of symptoms, acquisition of infections and other complications. As such, SCID meets the necessary criteria and is a suitable candidate for population-based newborn screening, and this has been borne out in prospective screening trials [5,6].

3. The Present: Screening for T and B Cell Lymphopenia

A seminal paper was published by Chan and Puck in 2005, describing the T-cell receptor excision circles (TREC) assay for the detection of SCID in a newborn screening setting [7]. TREC are small, circular pieces of episomal DNA which are produced during T cell receptor (TCR) rearrangement in naïve T cells, which serve as a surrogate marker of recent thymic emigrants. TREC copy numbers, measurable by quantitative reverse transcription polymerase chain reaction (qRT-PCR), are markedly reduced or absent in infants with SCID and other forms of T cell lymphopenia. Screening for SCID has become part of routine newborn screening programs in all US states, the District of Columbia and the Navajo Nation (Jeffrey Modell Foundation, http://www.info4pi.org). Several countries in Europe, Australasia and the Middle East have also commenced SCID screening programs, or are evaluating these in prospective studies (Table 1).

Table 1. Worldwide status of newborn screening programs for primary immunodeficiency diseases (PID). TREC: T-cell receptor excision circles; KREC: kappa-recombining exclusion circles; ADA: adenosine deaminase.

Country	Screening Strategy	Date of Commencement	Reference
United States of America **All 50 States** **District of Columbia** **Navajo Nation**	TREC	2008 (Wisconsin) National implementation	Dorsey & Puck 2017 [8] http://www.info4pi.org/
Italy **Tuscany** **Umbria** **Florence**	TREC/ADA	2010: Pilot study 2010: Pilot study 2013: Pilot study	http://ipopi.org/

Table 1. *Cont.*

Country	Screening Strategy	Date of Commencement	Reference
Taiwan	TREC	2010: Pilot study 2012: National implementation	Chien YH et al., 2017 [9]
Israel	TREC	2011: Pilot study 2015: National implementation	Rechavi et al., 2017 [10]
The Netherlands	TREC	2012: Pilot study 2015: Application approved	Blom et al., 2017 [11] http://ipopi.org/
Qatar	TREC	2012: National implementation	http://ipopi.org/
Germany	TREC/KREC	2013: Pilot study Application in progress	http://ipopi.org/
Sweden	TREC/KREC	2013: Pilot study	Barbaro et al., 2016 [6]
Japan	TREC/KREC	2014: Pilot study	http://ipopi.org/
France	TREC	2014: Pilot study	Audrain et al., 2014 [12] http://ipopi.org/
Spain **Andalucia**	TREC/KREC	2014: Pilot study	de Felipe et al., 2016 [13] http://ipopi.org/
Norway	TREC	2015: Pilot study	http://ipopi.org/
Puerto Rico	TREC	2016: National implementation	Dorsey & Puck 2017 [8] http://www.info4pi.org/
New Zealand	TREC	2017: Due to commence	http://ipopi.org/
Canada **Ontario** **British Columbia** **Yukon** **Prince Edward Island** **Nova Scotia** **New Brunswick**	TREC	Screening underway Approved, pending funding Approved, pending funding Approved, pending funding Approved, pending commencement Approved, pending commencement	http://ipopi.org/
Brazil	TREC	Pilot study	Kanegae et al., 2016 [14]
Denmark	TREC	Application in progress Pilot study	http://ipopi.org/
Iceland	TREC/KREC	Application in progress Pilot study	http://ipopi.org/
Iran	TREC/KREC	Pilot study	Personal communication
Saudi Arabia	TREC/KREC	Pilot study	http://ipopi.org/
Slovenia		Pilot study Application in progress	http://ipopi.org/
Turkey	TREC/KREC	Pilot study	Personal communication
United Kingdom	TREC	Application in progress Pilot study	http://ipopi.org/
Australia		Application in progress	http://ipopi.org/
Austria	TREC/KREC	Application in progress	http://ipopi.org/
Belgium **Flanders**	TREC/KREC	Application in progress Application in progress	Personal communication http://ipopi.org/
Czech Republic	TREC/KREC	Application in progress	Personal communication
Poland		Application in progress	http://ipopi.org/
Portugal		Application in progress	http://ipopi.org/
Romania		Application in progress	http://ipopi.org/
Switzerland	TREC/KREC	Application in progress	http://ipopi.org/
United Arab Emirates		Applications in progress	http://ipopi.org/

In addition to the detection of TREC levels as surrogate markers for thymic T cell output, it is also possible to evaluate B cell production by quantifying kappa-recombining excision circles (KREC), which are produced by similar mechanisms during rearrangement of the variable, diversity and joining domains (V(D)J recombination) of the B cell immunoglobulin kappa gene [15]. A multiplexed assay measuring TREC and KREC, along with beta-actin levels as a control for DNA quantity provides a strategy for simultaneous screening for T and B cell lymphopenia [16].

Screening for PID by measuring both TREC and KREC offers advantages over TREC screening alone. This includes identification of patients with X-linked agammaglobulinemia (XLA), an antibody deficiency disorder caused by mutations in the *BTK* gene which is essential for B cell development. Consequently, there is an absence of B cells, lack of antibody production, and severe infections with bacteria and other pathogens [17–19]. Mutations in other genes involved in B cell development have also been described, giving rise to an XLA-like disease with a similar phenotype [20]. Both groups of patients can be identified by low or absent KREC levels. Early identification of this disease is important, as it facilitates timely commencement of gammaglobulin replacement therapy. In addition to XLA, multiplexed TREC/KREC screening also facilitates diagnosis of individuals with late onset adenosine deaminase (ADA) deficiency, some cases of Nijmegen breakage syndrome (NBS) and other forms of PID which may otherwise be missed [16]. In the case of SCID, combined screening aids in the diagnostic process and guides targeted molecular evaluation, as different mutations will give rise to a variable pattern of T and B cell deficiency. Potential increased costs associated with KREC screening in addition to TREC screening have been suggested as a disadvantage of combined screening. Intrinsic costs of laboratory testing, patient follow-up and second tier testing must all be considered. However, the additional costs of adding KREC quantitation to a TREC-only platform are negligible, estimated to be less than €0.10 per test. Another proposed disadvantage of combined TREC/KREC screening was a higher recall rate for abnormal KREC levels. However, results of our recent evaluation of the combined TREC/KREC newborn screening program in Sweden show that the recall rate is dependent upon cut off levels set, but the recall rate in our cohort was similar to that for TREC-only testing programs [6], suggesting that this was unlikely to be an issue. There are some limitations of TREC-based screening. Some cases of SCID will not be identified, in the case where T cells are present but have abnormal function, or where the molecular defect lies downstream of TCR rearrangement (including Zap70 Deficiency, major histocompatibility complex (MHC) class II deficiency and some cases of delayed-onset adenosine deaminase (ADA) deficiency) [21–26].

4. The Future: Screening for Other Forms of PID

In addition to screening for immunodeficiency diseases that manifest with T and B cell lymphopenia, expanded screening for the detection of other diseases such as complement deficiencies and granulocyte disorders using protein-based assays has also been proposed. Disorders of granulocyte number and function give rise to severe, recurrent bacterial and fungal infections, and one possible method to identify these diseases involves measurement of granulocyte-specific proteins. The complement system consists of a large number of interacting components. Individuals with complement deficiency present variably, with significant bacterial infections caused by specific pathogens, or severe autoimmune or renal disease. In these patients, complement protein levels are reduced as a result of the underlying genetic mutation. It has been demonstrated that complement proteins can be eluted from DBS samples, allowing identification of C2- and C3-deficient patients at birth who have low or absent protein levels [27,28]. This can ultimately be expanded to develop multiplexed assays which enable detection of multiple complement cascade components and granulocyte specific proteins to enable detection of these disorders in the neonatal period, which would enable early diagnosis and treatment, minimizing long-term complications in these patients. Screening for granulocyte disorders and complement deficiency will be covered elsewhere in greater detail in this special issue on newborn screening for PID.

Targeted genetic testing is another newborn screening approach, and this is currently employed in screening algorithms for selected diseases such as cystic fibrosis [29]. Familial hemophagocytic lymphohistiocytosis (FHL) is a primary immunodeficiency disorder manifesting with a life-threatening inflammatory response secondary to impaired lymphocyte cytotoxicity. Several causative genetic mutations have been identified [20]. In Scandinavia, 50% of FHL cases are due to homozygous *UNC13D* inversion mutations, and a screening strategy based on the detection of reduced *UNC13D* wild type gene copy numbers was demonstrated to be an effective method by which to identify affected

individuals [30]. This approach could be expanded to enable identification of mutations in other FHL genes, and may have a role in screening for other disorders.

5. The Future: The Role of Next-Generation Sequencing in Newborn Screening for PID

Since completion of sequencing of the human genome project in 2003, advances in next-generation sequencing have progressed exponentially, resulting in an increased availability of testing, improved bioinformatic pipelines for data analysis, a faster turn-around time and a decrease in associated costs. This makes next-generation sequencing (NGS) an attractive and affordable modality to employ not only in a diagnostic setting, but also as a potential screening tool. Given that there is no single or multiplexed screening assay which can reliably detect all forms of PID at birth, we propose a screening approach for PID where all newborns undergo whole genome sequencing (WGS), with rapid analysis of the currently known 300+ genes, which would be expanded to include additional clinically relevant genes as they are identified.

Current strategies for newborn screening include metabolic assays, TREC/KREC quantification and targeted genetic studies, and this list is expected to expand. Second- and third-tier testing is required to confirm the findings of the screening test, and to make a specific diagnosis. Targeted mutation analysis or next-generation sequencing, including whole exome sequencing (WES) and whole genome sequencing, is often required to determine the underlying molecular diagnosis. At the Center for Metabolic Diseases (CMMS) at the Karolinska University Hospital in Stockholm, an aberrant result on first-tier metabolic testing that cannot be rapidly confirmed with a second-line test is further evaluated by genetic testing by WGS. This enables specific mutations to be identified in known disease-causing genes. If no mutation is found, parents are counselled and consented to undergo further genomic evaluation [31]. In this model, an aberrant test is followed up with WGS. As such, the next logical step in screening methodology would be to screen newborns for a range of genetic mutations using up-front NGS. With targeted sequencing being of limited value and the technical limitations of WES, WGS seems the most appropriate choice.

Pavey et al. recently reported the results of WGS-based screening for PID in a cohort of 1349 newborn and parent trios, who were analyzed for variants in 329 known immunodeficiency genes [32]. A genotype-first pipeline resulted in identification of 396 newborns with pathogenic/likely pathogenic mutations, however on further analysis, only one individual was found to have a genomically predicted immunodeficiency (complement component C9 deficiency). C9 deficiency is associated with an increased risk of life-threatening Neisserial infection [20], and early institution of prophylactic measures including immunization are of utmost importance in this patient group. A phenotype-first pipeline was also applied, in which 29 infants were identified to have clinical features suggestive of possible immunodeficiency, but did not have an identifiable mutation in any of the interrogated PID genes. Three of these children had pathogenic mutations in other (non-PID) genes which correlated clinically. No additional causative variants were found in the other children, the majority of whom were considered to be unlikely to have a PID [32]. These findings are consistent with results of a survey conducted by Boyle et al., who determined a PID prevalence of 1 in 1200 in the US [33]. Taken together, it is expected that screening 1000 newborns will identify one case of PID, however utilizing a NGS approach enables identification of other disorders. This is the first reported study evaluating genomic testing for PID as a first-line screening strategy in newborns. Longer-term clinical follow-up in cohorts such as these, and larger studies are required to determine test characteristics such as sensitivity and specificity.

The National Institutes of Health are currently undertaking the 'Newborn Sequencing in Genomic Medicine and Public Health' (NSIGHT) project which will evaluate the role of up-front genomic testing in neonates (https://www.genome.gov). These projects will include evaluation of exome sequencing in newborn screening for currently screened and additional disorders, and investigate the role of rapid turnaround genomic sequencing in the neonatal intensive care unit setting. They will also address key ethical, legal and social implications of implementation of such programs. This will involve

evaluating parental experiences related to receiving clinical information arising from genomic testing in the newborn period, and medical practitioner experiences in terms of utilizing genomic information and its impact upon clinical care delivery [34].

The notion of performing WGS on every infant at birth is controversial, and there are several factors that need to be considered regarding this potential approach to screening. The Global Alliance Paediatric Task Team recently published a series of recommendations regarding this screening approach, highlighting clinical utility, management of incidental findings, cost-effectiveness, data management, and ethical, legal and social implications as key areas for consideration [35].

5.1. Identification of Candidate Diseases and Genes to Be Evaluated in Newborn Genomic Screening Programs

The identification of key diseases for inclusion in genomic-based screening programs, selection of appropriate gene candidates for interrogation, and establishment of a testing panel are major considerations. In terms of screening for PID, an approach similar to that adopted by Pavey et al. [32] involving interrogation of a gene panel inclusive of the currently-described 300+ PID genes is an appropriate starting point. However, such panels would need to be regularly updated to include newly identified genes. It is probable that patients without identifiable molecular causes for their disease will have mutations in novel genes, and hence frequent revision of gene panels will enhance our future diagnostic capabilities. Although we advocate for the use of WGS as a screening tool for all newborns, this must proceed with caution. More than 8000 Mendelian diseases are currently recognized (https://omim.org/), however, initial evaluation in a WGS-based newborn screening program should only involve interrogation of genetic mutations which have a definite disease-causing role and where a clear genotype/phenotype correlation exists. However, it is expected that over time, there will be an increase in the number of screened genetic mutations and diseases.

In order to gain an appreciation of which disorders were likely to be significant causes of child mortality, and hence, possible disease candidates for newborn screening, we evaluated the causes of death in children less than 18 years of age in Sweden between 1987 and 2015 (Figure 1). There were 19,957 deaths during this time period. Seventy-six deaths (0.38%) were attributable to a known primary immunodeficiency disorder. Of these 76 cases, one death was attributed to common variable immunodeficiency, 4 to PID with predominant antibody deficiency, 5 to PID in combination with other major defects, 13 to combined immunodeficiency (including SCID), and 53 were attributed to unspecified immunodeficiency diseases. In addition, 849 deaths (4.3%) were due to infectious diseases and their complications, and it is likely that there were a considerable number of additional cases of undiagnosed PID in this group. Tambe et al. evaluated causes of child and infant mortality in the United Kingdom between 2006 and 2008, and found a mortality rate due to diseases of the blood, blood-forming organs and the immune system of 1.8 per 1,000,000 children in the 5–15 year age group [36], and 3.3 per 100,000 children aged less than 5 years [37]. Infectious diseases accounted for 9.5 deaths per 1,000,000 in the 5–15 year age group [36], and 27.9 per 100,000 neonates and 36 per 100,000 children aged 28 days to 4 years [37]. There are some limitations of the studies performed by our group and Tambe et al., given the degree of subjectivity in International Classification of Diseases (ICD) code assignment and potential categorization of a disease in different groups, and some cases might not be captured correctly due to a lack of detailed coding [23]. In addition, this type of analysis does not capture the significant morbidity associated with survivorship of severe diseases such as PID. Evaluation of such data facilitates identification of key diseases that result in death or significant morbidity in infancy and childhood, which should therefore be prioritized as candidates for inclusion in population-based newborn screening programs.

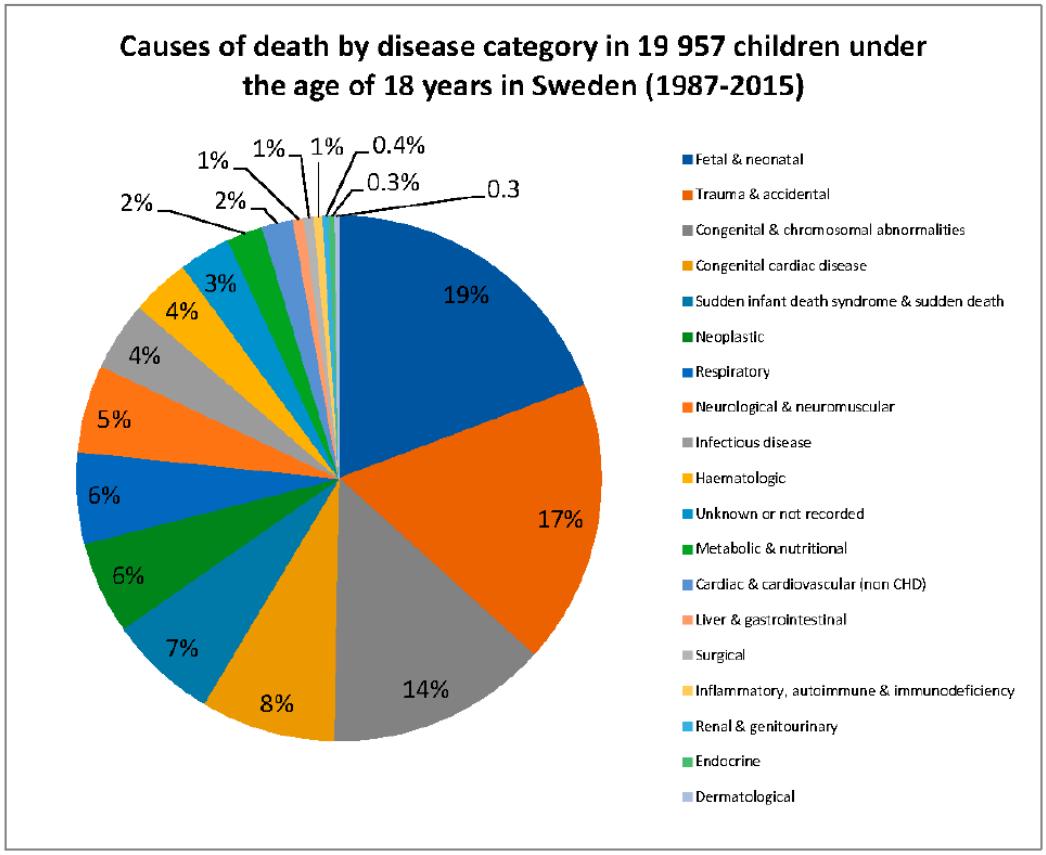


Figure 1. Causes of death by disease category in 19,957 children under the age of 18 years in Sweden (1987–2015).

5.2. Establishment of Robust and Cost-Effective Genomic Testing Systems

As for any diagnostic or screening assay, test characteristics must be agreeable, and an appropriate screening system must be established. All tests should be sufficiently sensitive and specific. A robust pipeline would be required, with a plan in place for data management, analysis and storage. Systems must also be established for managing abnormal results, with seamless integration with clinical services and timely access to clinical review, further testing, and management. A fast turn-around time, from sample collection to release of results is also essential to avoid any diagnostic delays. Furthermore, the process must be cost effective. A formal economic analysis is required to demonstrate that this approach is more cost effective than conventional screening strategies. There has been a significant decrease in assay-specific costs over time. Illumina, Inc. predict that in the near future, they will provide a WGS platform that will cost less than USD100 per genome (https://www.illumina.com).

5.3. Ethical, Legal and Social Implications

Population-based NGS screening raises several ethical, legal and social issues that must be considered, and are not discussed here in detail. These considerations represent a key area which will be addressed through the NSIGHT projects and similar studies evaluating newborn screening using genomic testing. In many countries, newborn screening is offered as a standard of care, and consent is typically obtained via an 'opt out' process. A change in approach to NGS screening would lead to an added level of consent that needs to be obtained from families given the implications of genetic testing. There is a risk that this could potentially deter families from engagement in screening

programs. Genetic data storage and management, biobanking of genetic material and its implications for other uses must also be considered. Variants of unknown significance are commonly found in NGS analyses. A clear plan must be established for the identification and reporting of such results. In addition, the finding of 'unexpected' or unrelated genetic mutations may have far-reaching effects on the individual and their family. The potential for identification of mutations in genes known to lead to debilitating or lifespan-reducing disease later in life for which no treatment exists must be considered, and how this will be managed should also be considered.

5.4. A New Model for Newborn Screening

Population-based NGS screening strategies represent a reversed approach to current screening practices, where typically a marker of disease is identified by a screening test, and then confirmed by genetic analysis. It stands to be determined if an upfront NGS testing approach will ultimately save time or money, given that functional tests may then be needed to confirm the genotype–phenotype correlation. This also raises the question as to if NGS screening will ultimately replace current screening tests, or if they would continue to be run in tandem. It is anticipated that the results of prospective genomic-based newborn screening programs will answer many of these questions, and inform future practice.

6. Conclusions

In the past, there was no method by which to identify infants with severe forms of PID, resulting in delayed diagnosis and significant complications. At the present time, newborn screening for the detection of severe forms of PID manifest by T and/or B cell lymphopenia using TREC or TREC/KREC screening has been established in many countries. There is the potential for future expansion to employ other screening modalities, such as protein-based assays and targeted genetic sequencing to enable identification of other forms of PID. However, it is not currently possible to screen for a wide range of diseases simultaneously using a single modality. It seems logical that the most effective and efficient way to screen newborns for an extensive list of diseases, which differ in terms of classification, pathophysiology and manifestation is using an up-front NGS testing strategy. Prior to adopting a genomic screening approach, selection of appropriate gene candidates for inclusion in screening panels, program structure, laboratory-clinical pipeline establishment, ethical, legal, social and financial implications must be both considered and evaluated in large, prospective studies to inform decisions regarding future implementation.

Acknowledgments: Jovanka King is a recipient of the Mike and Carole Ralston Travelling Fellowship 2016 from the RCPA Foundation.

Author Contributions: Jovanka King, Jonas F. Ludvigsson and Lennart Hammarström wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Itan, Y.; Shang, L.; Boisson, B.; Patin, E.; Bolze, A.; Moncada-Velez, M.; Scott, E.; Ciancanelli, M.J.; Lafaille, F.G.; Markle, J.G.; et al. The human gene damage index as a gene-level approach to prioritizing exome variants. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 13615–13620. [CrossRef] [PubMed]
2. Pai, S.Y.; Logan, B.R.; Griffith, L.M.; Buckley, R.H.; Parrott, R.E.; Dvorak, C.C.; Kapoor, N.; Hanson, I.C.; Filipovich, A.H.; Jyonouchi, S.; et al. Transplantation outcomes for severe combined immunodeficiency, 2000–2009. *N. Engl. J. Med.* **2014**, *371*, 434–446. [CrossRef] [PubMed]
3. Guthrie, R.; Susi, A. A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. *Pediatrics* **1963**, *32*, 338–343. [PubMed]
4. Wilson, J.; Jungner, G. *The principles and Practice of Screening for Disease*; World Health Organization: Geneva, Switzerland, 1968.

5. Kwan, A.; Abraham, R.S.; Currier, R.; Brower, A.; Andruszewski, K.; Abbott, J.K.; Baker, M.; Ballow, M.; Bartoshesky, L.E.; Bonilla, F.A.; et al. Newborn screening for severe combined immunodeficiency in 11 screening programs in the United States. *JAMA* **2014**, *312*, 729–738. [CrossRef] [PubMed]
6. Barbaro, M.; Ohlsson, A.; Borte, S.; Jonsson, S.; Zetterström, R.H.; King, J.; Winiarski, J.; von Döbeln, U.; Hammarström, L. Newborn screening for severe primary immunodeficiency diseases in sweden-a 2-year pilot trec and krec screening study. *J. Clin. Immunol.* **2017**, *37*, 51–60. [CrossRef] [PubMed]
7. Chan, K.; Puck, J.M. Development of population-based newborn screening for severe combined immunodeficiency. *J. Allergy Clin. Immunol.* **2005**, *115*, 391–398. [CrossRef] [PubMed]
8. Dorsey, M.; Puck, J. Newborn screening for severe combined immunodeficiency in the US: Current status and approach to management. *Int. J. Neonatal Screen.* **2017**, *3*, 15. [CrossRef]
9. Chien, Y.-H.; Yu, H.-H.; Lee, N.-C.; Ho, H.-C.; Kao, S.-M.; Lu, M.-Y.; Jaing, T.-H.; Lee, W.-I.; Chang, K.-W.; Shieh, C.-C.; et al. Newborn screening for severe combined immunodeficiency in Taiwan. *Int. J. Neonatal Screen.* **2017**, *3*, 16. [CrossRef]
10. Rechavi, E.; Lev, A.; Saraf-Levy, T.; Etzioni, A.; Almashanu, S.; Somech, R. Newborn screening for severe combined immunodeficiency in Israel. *Int. J. Neonatal Screen.* **2017**, *3*, 13. [CrossRef]
11. Blom, M.; Pico-Knijnenburg, I.; Sijne-van Veen, M.; Boelen, A.; Bredius, R.G.M.; van der Burg, M.; Schielen, P.C.J.I. An evaluation of the trec assay with regard to the integration of scid screening into the Dutch newborn screening program. *Clin. Immunol.* **2017**, *180*, 106–110. [CrossRef] [PubMed]
12. Audrain, M.; Thomas, C.; Mirallie, S.; Bourgeois, N.; Sebille, V.; Rabetrano, H.; Durand-Zaleski, I.; Boisson, R.; Persyn, M.; Pierres, C.; et al. Evaluation of the T-cell receptor excision circle assay performances for severe combined immunodeficiency neonatal screening on guthrie cards in a french single centre study. *Clin. Immunol.* **2014**, *150*, 137–139. [CrossRef] [PubMed]
13. De Felipe, B.; Olbrich, P.; Lucenas, J.M.; Delgado-Pecellin, C.; Pavon-Delgado, A.; Marquez, J.; Salamanca, C.; Soler-Palacin, P.; Gonzalez-Granado, L.I.; Antolin, L.F.; et al. Prospective neonatal screening for severe T- and B-lymphocyte deficiencies in Seville. *Pediatr. Allergy Immunol.* **2016**, *27*, 70–77. [CrossRef] [PubMed]
14. Kanegae, M.P.; Barreiros, L.A.; Mazzucchelli, J.T.; Hadachi, S.M.; de Figueiredo Ferreira Guilhoto, L.M.; Acquesta, A.L.; Genov, I.R.; Holanda, S.M.; Di Gesu, R.S.; Goulart, A.L.; et al. Neonatal screening for severe combined immunodeficiency in Brazil. *J. Pediatr. (Rio J)* **2016**, *92*, 374–380. [CrossRef] [PubMed]
15. Nakagawa, N.; Imai, K.; Kanegane, H.; Sato, H.; Yamada, M.; Kondoh, K.; Okada, S.; Kobayashi, M.; Agematsu, K.; Takada, H.; et al. Quantification of kappa-deleting recombination excision circles in Guthrie cards for the identification of early B-cell maturation defects. *J. Allergy Clin. Immunol.* **2011**, *128*, 223–225. [CrossRef] [PubMed]
16. Borte, S.; von Döbeln, U.; Fasth, A.; Wang, N.; Janzi, M.; Winiarski, J.; Sack, U.; Pan-Hammarström, Q.; Borte, M.; Hammarström, L. Neonatal screening for severe primary immunodeficiency diseases using high-throughput triplex real-time PCR. *Blood* **2012**, *119*, 2552–2555. [CrossRef] [PubMed]
17. Bruton, O.C. Agammaglobulinemia. *Pediatrics* **1952**, *9*, 722–728. [PubMed]
18. Bruton, O.C. Agammaglobulinemia (congenital absence of gamma globulin); report of a case. *Med. Ann. Dist. Columbia* **1953**, *22*, 648–650. [PubMed]
19. Vetrie, D.; Vorechovsky, I.; Sideras, P.; Holland, J.; Davies, A.; Flinter, F.; Hammarstrom, L.; Kinnon, C.; Levinsky, R.; Bobrow, M.; et al. The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. *Nature* **1993**, *361*, 226–233. [CrossRef] [PubMed]
20. Picard, C.; Al-Herz, W.; Bousfiha, A.; Casanova, J.L.; Chatila, T.; Conley, M.E.; Cunningham-Rundles, C.; Etzioni, A.; Holland, S.M.; Klein, C.; et al. Primary immunodeficiency diseases: An update on the classification from the international union of immunological societies expert committee for primary immunodeficiency 2015. *J. Clin. Immunol.* **2015**, *35*, 696–726. [CrossRef] [PubMed]
21. Grazioli, S.; Bennett, M.; Hildebrand, K.J.; Vallance, H.; Turvey, S.E.; Junker, A.K. Limitation of TREC-based newborn screening for ZAP70 severe combined immunodeficiency. *Clin. Immunol.* **2014**, *153*, 209–210. [CrossRef] [PubMed]
22. Hauck, F.; Blumenthal, B.; Fuchs, S.; Lenoir, C.; Martin, E.; Speckmann, C.; Vraetz, T.; Mannhardt-Laakmann, W.; Lambert, N.; Gil, M.; et al. Syk expression endows human ZAP70-deficient CD8 T cells with residual TCR signaling. *Clin. Immunol.* **2015**, *161*, 103–109. [CrossRef] [PubMed]

23. Kuo, C.Y.; Chase, J.; Garcia Lloret, M.; Stiehm, E.R.; Moore, T.; Aguilera, M.J.; Lopez Siles, J.; Church, J.A. Newborn screening for severe combined immunodeficiency does not identify bare lymphocyte syndrome. *J. Allergy Clin. Immunol.* **2013**, *131*, 1693–1695. [CrossRef] [PubMed]
24. Lev, A.; Simon, A.J.; Broides, A.; Levi, J.; Garty, B.Z.; Rosenthal, E.; Amariglio, N.; Rechavi, G.; Somech, R. Thymic function in mhc class II-deficient patients. *J. Allergy Clin. Immunol.* **2013**, *131*, 831–839. [CrossRef] [PubMed]
25. Lyttle, A.; Roifman, C.; Dadi, H.; Wright, N.; Kavadas, F. MHC class II deficiency in the Dene native population: A case report highlighting pitfalls in diagnosis and treatment. *Allergy Asthma Clin. Immunol.* **2014**, *10*, A1. [CrossRef]
26. Speckmann, C.; Neumann, C.; Borte, S.; la Marca, G.; Sass, J.O.; Wiech, E.; Fisch, P.; Schwarz, K.; Buchholz, B.; Schlesier, M.; et al. Delayed-onset adenosine deaminase deficiency: Strategies for an early diagnosis. *J. Allergy Clin. Immunol.* **2012**, *130*, 991–994. [CrossRef] [PubMed]
27. Janzi, M.; Sjöberg, R.; Wan, J.; Fischler, B.; von Döbeln, U.; Isaac, L.; Nilsson, P.; Hammarström, L. Screening for C3 deficiency in newborns using microarrays. *PLoS ONE* **2009**, *4*, e5321. [CrossRef] [PubMed]
28. Hamsten, C.; Skattum, L.; Truedsson, L.; von Döbeln, U.; Uhlén, M.; Schwenk, J.M.; Hammarström, L.; Nilsson, P.; Neiman, M. Heat differentiated complement factor profiling. *J. Proteom.* **2015**, *126*, 155–162. [CrossRef] [PubMed]
29. Rosenfeld, M.; Sontag, M.K.; Ren, C.L. Cystic fibrosis diagnosis and newborn screening. *Pediatr. Clin. N. Am.* **2016**, *63*, 599–615. [CrossRef] [PubMed]
30. Borte, S.; Meeths, M.; Liebscher, I.; Krist, K.; Nordenskjold, M.; Hammarstrom, L.; von Dobeln, U.; Henter, J.I.; Bryceson, Y.T. Combined newborn screening for familial hemophagocytic lymphohistiocytosis and severe T- and B-cell immunodeficiencies. *J. Allergy Clin. Immunol.* **2014**, *134*, 226–228. [CrossRef] [PubMed]
31. Stranneheim, H.; Wedell, A. Exome and genome sequencing: A revolution for the discovery and diagnosis of monogenic disorders. *J. Intern. Med.* **2016**, *279*, 3–15. [CrossRef] [PubMed]
32. Pavey, A.R.; Bodian, D.L.; Vilboux, T.; Khromykh, A.; Hauser, N.S.; Huddleston, K.; Klein, E.; Black, A.; Kane, M.S.; Iyer, R.K.; et al. Utilization of genomic sequencing for population screening of immunodeficiencies in the newborn. *Genet. Med.* **2017**. [CrossRef] [PubMed]
33. Boyle, J.M.; Buckley, R.H. Population prevalence of diagnosed primary immunodeficiency diseases in the United States. *J. Clin. Immunol.* **2007**, *27*, 497–502. [CrossRef] [PubMed]
34. Berg, J.S.; Agrawal, P.B.; Bailey, D.B.; Beggs, A.H.; Brenner, S.E.; Brower, A.M.; Cakici, J.A.; Ceyhan-Birsoy, O.; Chan, K.; Chen, F.; et al. Newborn sequencing in genomic medicine and public health. *Pediatrics* **2017**, *139*, e20162252. [CrossRef] [PubMed]
35. Friedman, J.M.; Cornel, M.C.; Goldenberg, A.J.; Lister, K.J.; Sénécal, K.; Vears, D.F.; The Global Alliance for Genomics and Health Regulatory and Ethics Working Group Paediatric Task Team. Genomic newborn screening: Public health policy considerations and recommendations. *BMC Med. Genom.* **2017**, *10*, 9. [CrossRef] [PubMed]
36. Tambe, P.; Sammons, H.M.; Choonara, I. Child mortality of children aged 5–15 years in the UK and Sweden: A comparison. *Arch. Dis. Child.* **2016**, *101*, 409–410. [CrossRef] [PubMed]
37. Tambe, P.; Sammons, H.M.; Choonara, I. Why do young children die in the UK? A comparison with sweden. *Arch. Dis. Child.* **2015**, *100*, 928–931. [CrossRef] [PubMed]

CHAPTER 9: SUMMARY AND CONCLUSION

9.1 Introduction and Contextual Statement

This thesis sought to address gaps in the literature regarding optimal strategies for newborn screening for PID, and the application of novel approaches to enable identification of a wider range of diseases. Specifically, this thesis aimed to:

1. Determine the efficacy of a combined TREC/KREC screening approach for the identification of newborns with severe forms of PID manifested by T and/or B cell lymphopaenia
2. Determine the utility of screening assays beyond the newborn period, as part of the diagnostic evaluation of patients with suspected immunodeficiency
3. Establish a method for screening newborns for hypogammaglobulinaemia
4. Explore the role of novel genetic screening strategies to identify newborns with key disease susceptibility factors and facilitate the application of personalised medicine

Key findings arising from this thesis are summarised below, along with a discussion of the implications of these results. Final conclusions are drawn, and further questions arising from this work are presented with a view to informing future research efforts.

9.2 Summary of Key Findings

Seven key findings can be drawn from the research presented in this thesis.

1. Prospective newborn screening programs for PID have been established in several countries

- Based on published, prospective studies, 4,330,807 infants have been screened for PID in 4 countries
- Ninety-five infants with severe forms of PID have been successfully identified and treated in these studies
- Newborn screening for PID is seen as health priority worldwide, with many countries currently evaluating or piloting screening programs, or planning to commence screening in the near future

2. ***There remains a lack of consensus regarding the optimal testing strategy to screen newborns for PID***

- Most centres with established programs are screening infants for SCID as the target condition using a TREC-only assay
- Other approaches include combined TREC/KREC assays, or TREC/ADA deficiency screening using mass spectrometry

3. ***A multiplexed TREC/KREC/ACTB assay is an effective modality by which to screen newborns for PID manifested by T and/or B cell lymphopaenia, thereby confirming the efficacy of DNA-based screening strategies for PID***

- Three patients with severe forms of PID were identified in a cohort of 58,834 newborns screened over a two-year period, facilitating early diagnosis and treatment
- Most premature infants will return a normal TREC/KREC screening result, irrespective of gestational age
- There are several benefits of simultaneous screening for both T and B cell lymphopaenia using a multiplexed TREC/KREC assay, including identification of a broader range of immunodeficiency diseases

4. ***In addition to their utility as newborn screening tests, TREC/KREC assays have an important role beyond the neonatal period***

- KREC levels remain low or absent in patients with XLA throughout life, whereas TREC levels are comparable with those in healthy control subjects
- TREC/KREC assays therefore provide a useful adjunctive testing strategy in the evaluation of older children and adults with suspected PID
- The diagnosis of XLA is frequently significantly delayed beyond the ‘classic’ 6-12 month age of presentation, providing further evidence supporting the case for screening for congenital B cell deficiency disorders

5. ***An immunoglobulin heavy chain gene expression assay performed on RNA extracted from dried blood spots can identify children with complete antibody deficiency disorders such as XLA and X-HIGM syndrome, providing a novel approach to screening newborns for hypogammaglobulinaemia***

- Expression of the immunoglobulin heavy chain genes is undetectable in patients with XLA and X-HIGM syndrome

- Immunoglobulin heavy chain gene expression is variable in healthy controls and patients with PID who have normal or reduced immunoglobulin levels
- RNA can be successfully extracted from DBS up to 17 years of age using our method

6. Newborns can be screened for single nucleotide polymorphisms which predispose to certain disease states, providing opportunities for personalised and precision medicine

- Newborns can effectively be screened for SNPs in the fucosyltransferase (*FUT2* and *FUT3*) genes, which determine the expression of histo-blood group antigens (HBGA) (H type 1 and Lewis) with implications for predisposition to specific infectious and inflammatory diseases
- A higher than expected proportion of newborns in the cohort were found to have polymorphisms in the *FUT2* and *FUT3* genes resulting in an increased proportion of non-secretors and Lewis b negative individuals with ensuing predisposition to specific infections and other diseases
- If available in the early neonatal period, information regarding genetic polymorphisms may be used to inform tailored health care decisions regarding vaccination, screening and therapy for specific diseases

7. There is potential to further expand newborn screening to enable detection of a broader range of primary immunodeficiency diseases

- Future approaches include protein-based assays and targeted sequencing strategies
- Up-front NGS (whole exome or whole genome sequencing) is a testing strategy which would enable screening of the largest number of additional forms of PID through identification of mutations in all currently known PID-associated genes
- Genotype-phenotype correlation, clinical and laboratory pipelines, costs, management of variants of unknown significance along with ethical, legal and social implications of an NGS-based screening program must be evaluated prior to adopting this approach

9.3 Discussion

The aim of newborn screening programs is to identify infants with severe diseases, which are asymptomatic at birth, and for which effective treatment exists[39]. Primary immunodeficiency diseases meet these and other criteria for inclusion as screened diseases[39], given the potentially devastating effects of untreated disease. It is well documented that early identification of severe forms of PID such as SCID and rapid commencement of therapy results in reduced morbidity and mortality[6].

Screening for PID has been embraced as a health care priority around the world, with reports outlining prospective screening programs, pilot studies and applications to commence programs in several regions (Paper V). At the time of publication of Paper I, four centres had published the results of their prospective programs, where cumulatively 4,330,807 infants had been screened and 95 infants were diagnosed with severe forms of PID. The number of screened infants worldwide continues to increase exponentially as more centres establish PID screening programs.

Despite the uptake of newborn screening programs for PID in several countries worldwide, the ideal screening strategy for PID remains controversial. In fact, there is great variability in newborn screening practices in general between different countries, and even between different centres within the same country (Paper V). There are several factors determining the choice of screened diseases in any given centre, impacted upon by issues such as disease prevalence, available methodologies and instruments, and economic considerations[40-42]. TREC-only screening programs enable identification of PID manifested by T cell lymphopaenia, however the inclusion of KREC quantitation has the additional benefit of detecting disorders associated with B cell lymphopaenia[10]. Paper II presents the findings of the largest prospective newborn screening study utilising a multiplexed TREC/KREC/ACTB assay, confirming the efficacy of DNA-based screening strategies for PID. Three infants with PID were identified during this two-year program, and, to our knowledge, no infants with severe forms of PID were missed.

It is important to note that there are some limitations to currently available screening assays. TREC assays will detect most, but not all forms of SCID. Infants with T cell functional defects (with preserved number), SCID due to functional anomalies downstream of the T cell receptor (e.g. Zap 70 deficiency) or MHC Class II deficiency, and some cases of late-onset ADA deficiency, will not be identified by this assay[43-45]. The benefits of multiplexed TREC/KREC assays include the ability to screen for additional disorders that would otherwise be missed, including congenital B cell deficiency disorders, such as XLA and autosomal recessive, XLA-like disorders. Although XLA is not generally considered to be as ‘urgent’ to treat (as compared with SCID), it is worth noting that the diagnosis of this condition is frequently significantly delayed, as demonstrated in Paper III. This delay results in the evolution of complications of severe, untreated infection, such that by the time of diagnosis, patients have already developed bronchiectasis and other irreversible comorbidities[17]. Identifying infants with these conditions in the newborn period would therefore enable commencement of gammaglobulin replacement therapy prior to onset of symptoms.

KREC assays were initially established to mark the replication history of B cells and assess B cell recovery following HSCT[11,46], prior to their application as a newborn screening assay[47].

The potential clinical utility of TREC/KREC assays in the diagnostic work-up of patients with suspected PID who were not screened at birth was demonstrated (Paper III). We showed that KREC levels remain low or absent throughout life in patients with XLA and XLA-like disease, and hence, this test may be a useful adjunct to current laboratory tests in the clinical evaluation of patients beyond the newborn period. The utility of TREC/KREC assays in the sub-classification of patients with CVID has been evaluated. One group classified 40 patients according to their TREC and KREC levels, and found an association between these levels and disease severity[13]. Two subsequent studies found reduced levels in patients compared with controls, however levels did not correlate with specific disease phenotypes[14,15]. The assay may assist in differentiating between patients with CVID and CID[13,14]. Given that TREC/KREC testing can be performed on stored DNA or DBS specimens, this overcomes the need to collect fresh blood specimens and simplifies sample transport between laboratories in situations where this is challenging.

Newborn immunoglobulin levels measured by nephelometry or other protein-based quantitation techniques reflect maternal levels, and therefore these methods are not suitable to determine an infant's endogenous capacity to produce antibodies[20]. Hypogammaglobulinaemia may be due to a primary antibody deficiency disorder, or may be a feature of other forms of PID[1], and hence an ability to screen for this has the potential to identify infants with a variety of immunodeficiency diseases. We developed a method by which to extract RNA from DBS specimens, and evaluate expression of the immunoglobulin heavy chain genes (*IGHG1*, *IGHA1*, *IGHM*) (Chapter 6). We demonstrated a proof of concept that this method could effectively identify children with complete failure of antibody production (i.e., XLA and X-HIGM syndrome) who had absent expression of these genes. We performed this assay on the original DBS of one newborn with a known family history of XLA, providing additional evidence that this approach could be used as a screening test for hypogammaglobulinaemia. The currently available KREC assay would have enabled identification of the infants with XLA, and hence the gene expression assay would not offer any additional diagnostic benefit. However, as expected, children with X-HIGM syndrome have been demonstrated to have normal TREC and KREC levels[10] and hence would be missed on a standard TREC/KREC screening assay. This provides an example of how this transcriptomic-based assay could potentially increase our capacity to detect a broader range of disorders. This assay, also performed on healthy controls and patients with various forms of PID, provided a proof of concept that this strategy can be used to evaluate expression of the immunoglobulin gene transcriptome in health and disease. Although our assay in its present form is not sufficiently sensitive to detect more minor degrees of hypogammaglobulinaemia, we believe that with further refinement, this will be achievable in the near future. This assay may also have further applicability in the future to determine

endogenous antibody production in those patients in whom protein measurement is confounded, for example by maternal transfer (in infants) and gammaglobulin replacement therapy.

In addition to screening for specific disease states, we explored the utility of screening newborns for a series of SNPs which confer susceptibility to specific infectious and inflammatory diseases (Paper IV). This would therefore determine an individual's risk factors for disease, and facilitate provision of individualised recommendations regarding treatment, screening and preventative measures, such as vaccination. Polymorphisms in the *FUT2* and *FUT3* genes determine the expression of histo-blood group antigens (HBGA) (H type 1 and Lewis), which influence an individual's susceptibility to various diseases[29].

Of specific interest was the susceptibility to norovirus and rotavirus[30,48,49], and the implications this has for vaccine recommendations. We found that a higher than expected proportion of infants were non-secretors and Lewis b negative, with an increased predisposition to infections such as rotavirus. This data supports the importance of rotavirus vaccination and in settings where this is not readily available, information from this newborn screening genotyping assay could assist clinicians to make recommendations as to which children would benefit most from vaccination. This screening approach may be expanded in the future to identify susceptibility factors for other specific diseases. In addition, this approach could be used to screen for polymorphisms predisposing individuals to adverse drug reactions, thereby enabling specific recommendations regarding pharmacotherapy and avoidance of severe reactions. Performing these studies soon after birth would be advantageous in that a 'catalogue' of genetic susceptibility could be generated and called upon as required to guide management.

With well over 320 known molecular causes for PID[1] to date, there is no established single test to enable simultaneous detection of every disease. Currently available strategies focus on the identification of PID manifested by T and/or B cell lymphopaenia. However, other important forms of PID (including but not limited to granulocyte disorders, complement deficiencies and immune dysregulatory conditions) cannot be identified using these tests alone.

Making a diagnosis of PID is challenging in the current era, due to issues such as lack of awareness in the community and by medical practitioners, and variable or atypical presentations. There is frequently a delay in diagnosis, sometimes up to several years from the onset of symptoms[16]. We explored the future role of up-front NGS (whole exome or whole genome sequencing) as the ideal testing strategy which would enable screening of the largest number of additional forms of PID, through identification of mutations in all currently known PID-associated genes (Paper V). This approach represents a paradigm shift, as genetic studies have, until recently, been used to confirm the findings of screening tests. Up-front NGS

has been evaluated in the context of screening for PID and identified one case in 1349 newborns - a case of complement deficiency[28] which would not have been identified by other means. This provides further evidence for this approach to be considered to improve diagnostic capabilities. However, large, prospective studies are required to evaluate the associated technical aspects, feasibility, acceptability, ethical, legal and social issues[50]. It will also need to be determined whether NGS can exist as a stand-alone, up-front technique, or if other current strategies (such as TREC/KREC screening) will need to continue to be run in tandem, given that not all infants returning an abnormal screening test will have an identifiable molecular defect.

9.4 Conclusions and Future Directions

Since their inception in the 1960s, newborn screening programs have enabled early identification and provision of life-saving treatment for infants with severe diseases which are asymptomatic at birth. More recent technological advances have enabled expansion of current programs to include screening infants for severe forms of immunodeficiency, facilitating early treatment and reducing disease-related morbidity and mortality.

Although there have been significant, rapid advances in this field over recent years, and despite application of best available technology, we are still only able to identify a handful of the 320+ different molecular forms of PID. It is anticipated that newborn screening practices for PID will continue to evolve and expand in the future, enabling detection of an increased number of diseases. It is expected that modalities such as NGS will have a more prominent role as costs, turn-around times and technical factors become more acceptable.

This work also poses several new lines of enquiry to be addressed by future research. For example, what is the aetiology of T cell lymphopaenia and combined immunodeficiency in infants who return a positive screening test, who do not have a molecular diagnosis? What is their prognosis and clinical course? Where do the answers lie regarding pathogenesis of their disease? There are also questions raised regarding the most economically feasible approaches to screening for PID – is it cost-effective to screen infants in population-based screening programs in all countries using current DNA-based technologies? Is it cost-effective to screen for other (non-SCID) forms of PID? Will screening for other forms of PID save lives and decrease morbidity and mortality? Regarding future testing strategies, if we adopt NGS-based screening methodologies, will this be acceptable to the public? What are the wider implications in terms of ethical and legal issues? Will we still need to run supportive screening tests in parallel? Will we miss cases where there is a clear phenotype but no identifiable genetic abnormality? It is expected that our work in this field will continue, in an effort to answer some of these questions in the future.

In conclusion, the findings reported in this thesis contribute to the growing experience and literature regarding newborn screening for PID. This work provides further evidence to support the case for newborn screening for these significant conditions, and also presents potential strategies which could be employed in order to advance our current screening capabilities. The ultimate goal of newborn screening programs for immunodeficiency is not only to save lives, but also to improve the quality of life of affected patients and their families.

APPENDICES

Appendix I: Presentation Abstracts

I. **'Newborn screening for primary immunodeficiency: The next generation'**
Abstract published in Pathology (2018) 50(S1), p. S29.
https://doi.org/10.1016/j.pathol.2017.12.066
Invited speaker, Genetics Stream
Royal College of Pathologists of Australasia (RCPA) Pathology Update Meeting, Sydney, New South Wales, March 2018

II. **'Newborn screening for primary immunodeficiency: TREC, KREC and beyond'**
Abstract published in Pathology (2018) 50(S1), p. S45.
https://doi.org/10.1016/j.pathol.2017.12.108
Invited speaker, Immunology Stream
Royal College of Pathologists of Australasia (RCPA) Pathology Update Meeting, Sydney, New South Wales, March 2018

III. **'Case studies in paediatric primary immunodeficiency'**
Invited speaker
Australasian Society for Clinical Immunology and Allergy (ASCIA) SA Branch Meeting, February 2018

IV. **'Newborn screening for primary immunodeficiency diseases'**
Invited speaker
SA Pathology Colloquium, Adelaide
November 2017

V. **'Newborn screening for primary immunodeficiency'**
Invited speaker
Australasian Society for Clinical Immunology and Allergy (ASCIA) SA Branch Meeting, April 2017

Pathology (2018) **50(S1)**, pp. S29–S32

Genetics

FINDING GOLD IN FEINGOLD SYNDROME-2

Cheng Yee Chan[1], Jane Watt[1], Alex Magee[2], Kate Gibson[2], Richard King[1]
[1]*Genetics Laboratory, Canterbury Health Laboratories, Christchurch, and* [2]*Genetic Health Services NZ, South Island Hub, Christchurch, New Zealand*

Feingold syndrome-2 (MIM 614326) is a rare autosomal dominant disorder, characterised by a variable combination of short stature, microcephaly, digital anomalies and learning difficulties. To date, only 12 cases have been reported in literature, with the smallest region of overlap (SRO) encompassing MIR17HG and the first exon of GPC5. Although previous work with mouse models has demonstrated that MIR17HG haploinsufficiency produces a phenotype consistent with the skeletal features of Feingold syndrome-2, the contribution of *GPC5* haploinsufficiency to the phenotype could not previously be ruled out. This presentation highlights the first known case of Feingold syndrome-2 with a 13q31.3 deletion that solely involves the *MIR17HG* gene and solidifies previous supportive evidence of *MIR17HG* pathogenicity in this disorder.

UTILISING GENOMICS AT THE BC CANCER AGENCY (BCCA) – MY FELLOWSHIP EXPERIENCE IN LYMPHOID CANCER RESEARCH

Yasmin Harvey[1,2]
[1]*Sullivan Nicolaides Pathology, Brisbane, Australia; and* [2]*Centre for Lymphoid Cancer, British Columbia Cancer Agency, Vancouver, Canada*

Advances in technology have enabled expansive research into genomic aberrations with a recent focus on cancer, including haematological malignancies. My research fellowship utilised the Nanostring platform to measure gene expression, and apply assays to formalin-fixed paraffin-embedded tissue (FFPET) samples from patients with classical Hodgkin lymphoma and diffuse large B cell lymphoma. Technical validation of varied custom Nanostring codesets, ranging from 20 to 800+ genes were performed. Epstein-Barr virus-encoded RNA2 (EBER2) gene expression was assessed against EBER in situ hybridisation in FFPET biopsies from patients with classical Hodgkin lymphoma.

The BCCA provides an impressive model for research efforts with a focus on delivering clinically useful research findings back into the clinic to impact patient management. Dedicated local collaborative efforts between patients, medical and scientific personnel and complimentary institutions have resulted in significant and continued contributions to lymphoma research. The RCPA Foundation Mike and Carole Ralston travelling fellowship provides a valuable opportunity to gain experience and insight into applications of genomics which is a rapidly growing, exciting area of medicine, particularly relevant to clinical diagnostic pathology.

NEWBORN SCREENING FOR PRIMARY IMMUNODEFICIENCY DISEASES: THE NEXT GENERATION

Jovanka King[1,2]
[1]*Department of Immunopathology, SA Pathology, Women's and Children's Hospital Campus; Robinson Research Institute, Discipline of Paediatrics, School of Medicine, University of Adelaide, North Adelaide, SA, Australia; and* [2]*Department of Clinical Immunology, Karolinska University Hospital Huddinge, Stockholm, Sweden*

Population-based newborn screening programs enable identification of infants with a range of severe diseases prior to the onset of symptoms, facilitating early treatment. Primary immunodeficiency diseases (PID) are a heterogeneous group of genetically determined inborn errors of immunity. Affected children present with severe, recurrent or unusual infections, and early diagnosis improves outcomes for affected patients.

To date, over 300 distinct genetic molecular abnormalities resulting in PID have been identified. Currently available newborn screening strategies enable identification of infants with severe forms of PID manifested by T and B cell lymphopenia, using PCR-based techniques to quantify T cell receptor excision circles (TREC) and KREC (kappa recombining exclusion circles).[1] However, this approach will not identify other forms of PID. Several approaches have been proposed to improve current PID screening capabilities, including application of protein based assays and gene copy number variation analyses.[2] Given the advances in -omics-based medicine, it is likely that other methodologies, including transcriptomic and genomic approaches will have an increasingly prominent role in newborn screening practices in the future.[2]

References

1. Borte S, von Döbeln U, Fasth A, *et al*. Neonatal screening for severe primary immunodeficiency diseases using high-throughput triplex real-time PCR. *Blood* 2012; 119: 2552–5.
2. King JR, Hammarström L. Newborn screening for primary immunodeficiency diseases: history, current and future practice. *J Clin Immunol* 2017; Nov 8: (Epub ahead of print).

DEVELOPING QUALITY ASSURANCE PROGRAM FOR TOTAL DNA EXTRACTION

Sze Yee Chai[1], Nalishia Pillay[1], Tony Badrick[1], Bruce Bennetts[2], Martin Horan[1]
[1]*Molecular Genetics, Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP), Sydney, and* [2]*Sydney Genome Diagnostics, The Children's Hospital at Westmead, Sydney, NSW, Australia*

The extraction of high quality genomic DNA is pivotal for all downstream genetic analyses. To assist laboratories in the quality assurance of their DNA isolation procedure, the RCPAQAP Molecular Genetics discipline developed a DNA extraction proficiency testing program in 2016 and invited six

Print ISSN 0031-3025/Online ISSN 1465-3931

USING QUALITY ASSURANCE TO DETERMINE UNCERTAINTY OF MEASUREMENT

Louise Wienholt[1], Kristie Chapman[1], Emma Dawson[1], Grace Moyo[1], Katrina Fryer[1], Tiffany Hughes[1,2]
[1]The Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP), Sydney, NSW, and [2]Flinders Medical Centre (FMC), Adelaide, SA, Australia

Within the context of pathology, the term 'uncertainty' is defined as the doubt of accuracy of a measurement. The commonly used term used to describe this concept is measurement uncertainty (MU), which characterises the dispersion of results about a single measurement. As such, MU provides a quantitative estimate of the variability in results a laboratory would normally expect if the measurement were to be repeated at another time. Medical pathology laboratories are required as part of ISO 15189 accreditation to determine or estimate the MU for all quantitative results.
Using data obtained from The Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP) the following aspects of MU will be discussed:

- The clinical importance of MU data.
- The immunological tests appropriate for UM calculations.
- Understanding how MU is derived referencing The National Pathology Accreditation Advisory Council (NPAAC) Requirements for the Estimation of Measurement Uncertainty (2007 edition).
- The optimal way in which this data should be presented to be clinically useful.

A REVIEW AND WHAT'S NEW IN RCPAQAP IMMUNOPATHOLOGY

Kristie Chapman[1], Louise Wienholt[1], Emma Dawson[1], Grace Moyo[1], Katrina Fryer[1], Tiffany Hughes[1,2]
[1]The Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP), Sydney, NSW, and [2]Flinders Medical Centre (FMC), Adelaide, SA, Australia

This session will summarise the current Immunology Programs available through the RCPAQAP; review the program changes that were implemented over 2017; and summarise the program changes planned for 2018.

DIAGNOSTICS IN PRIMARY IMMUNODEFICIENCY

Klaus Warnatz
Center for Chronic Immunodeficiency (CCI), University Medical center Freiburg, Freiburg, Germany

Primary immunodeficiencies (PID) belong to the rare diseases. Currently over three hundred different PID entities have been described. Due to the low prevalence and often variable presentation a high degree of suspicion has to be raised in order to avoid unnecessary and potentially harmful delay in diagnosis of the primary immunodeficiency. Simple, cost effective and readily available screening tests need to set the path for further diagnostic steps.

This presentation will enable you to learn the warning signs of different PIDs and to initiate the first diagnostic steps. In the second part, you will learn about the potential further steps taken at experienced diagnostic centres including specialised immunological tests as well as genetic work up by next generation sequencing. Using case reports, you will learn about the diagnostics of combined immunodeficiencies, antibody deficiencies, deficiencies of the innate immune system as well as haemophagocytic lymphohistiocytosis (HLH). Potential future developments in the diagnosis of different PID will be pointed out where appropriate.

NEWBORN SCREENING FOR PRIMARY IMMUNODEFICIENCY DISEASES: TREC, KREC AND BEYOND

Jovanka King[1,2]
[1]Department of Immunopathology, SA Pathology, Women's and Children's Hospital Campus; Robinson Research Institute and Discipline of Paediatrics, School of Medicine, University of Adelaide, North Adelaide, SA, Australia; and [2]Department of Clinical Immunology, Karolinska University Hospital Huddinge, Stockholm, Sweden

Primary immunodeficiency diseases (PID) are a heterogeneous group of genetically determined inborn errors of immunity, with affected children presenting with severe, recurrent or unusual infections. Severe combined immunodeficiency (SCID) is one form of PID which is fatal without definitive treatment with haematopoeitic stem cell transplantation. Evidence suggests that outcomes are markedly improved if this is instituted by 3.5 months of age[1] – a goal only achievable through early identification of affected infants through newborn screening.
Currently available screening strategies aim to identify newborns with severe forms of PID manifested by T and B cell lymphopenia use PCR-based techniques to quantify T cell receptor excision circles (TREC) and KREC (kappa recombining exclusion circles), which are surrogate markers for T and B cell production, respectively. TREC-alone and multiplexed TREC-KREC screening approaches have been described.[2] Newborn screening for PID is an evolving field, and technologies are being developed which may enable future identification of complement deficiencies, granulocyte disorders and immunoglobulin deficiencies, which cannot be identified using currently available strategies. Given the rapid advances in genomic medicine, next generation sequencing may have an increasingly prominent role in screening for PID and other diseases.[3]

References

1. Pai SY, Logan BR, Griffith LM, *et al.* Transplantation outcomes for severe combined immunodeficiency, 2000-2009. *N Engl J Med* 2014; 371: 434–46.
2. Borte S, von Döbeln U, Fasth A, *et al*. Neonatal screening for severe primary immunodeficiency diseases using high-throughput triplex real-time PCR. *Blood* 2012; 119: 2552–5.
3. King J, Ludvigsson J, Hammarström L. Newborn screening for primary immunodeficiency diseases: the past, the present and the future. *Int J Neonatal Screen* 2017; 3: 19.

ascia
australasian society of clinical immunology and allergy inc.

South Australian Branch

Meeting

Tuesday 20th Feb ,6.15 pm start

Venue

georges_____
on waymouth est 2002

20 Waymouth Street
Adelaide SA 5000

Educational Topic

Case Studies in Paediatric Immunodeficiency

Dr Jovanka King
Paediatric Immunologist and Immunopathologist

Dinner

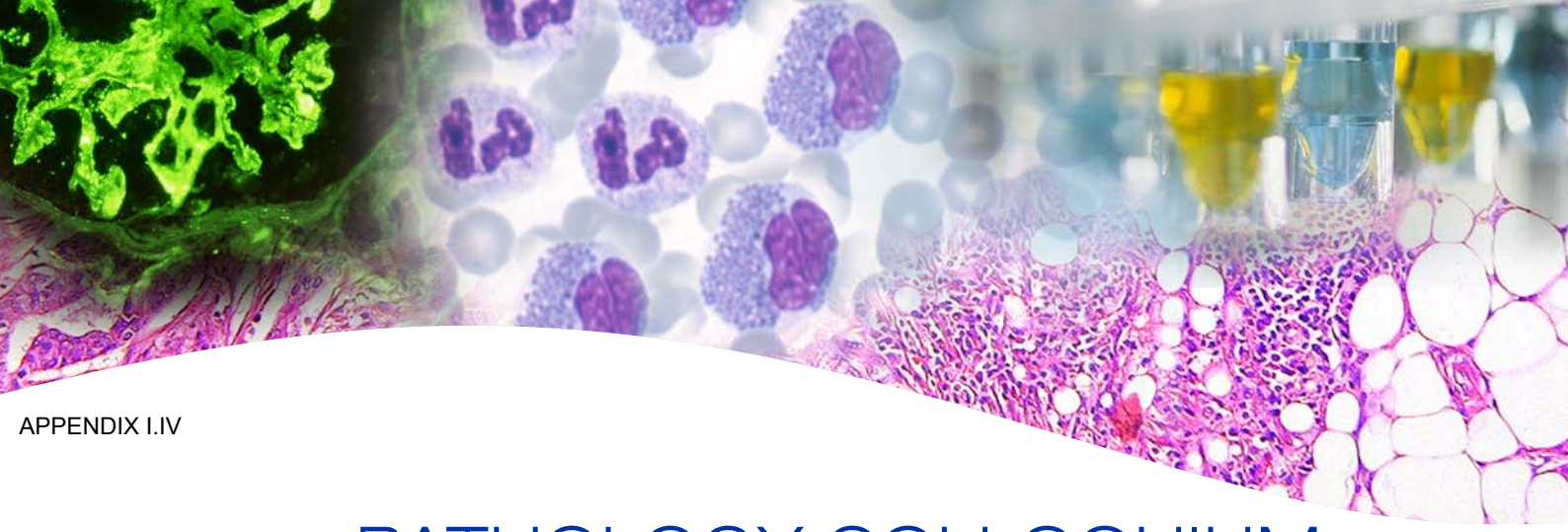
Will be served

Sponsored by:

Nestle Heath Science

Please RSVP by 5 pm Friday 16th Feb
Via "return" email to
Pravin.Hissaria@sa.gov.au

RSVP essential for dinner and early RSVP appreciated



PATHOLOGY COLLOQUIUM

Tuesday 28 November 2017, 5:00pm to 7:45pm
Jerningham Room, The Lion Hotel
161 Melbourne Street, North Adelaide

Program

5:00pm **LIGHT REFRESHMENTS**

5:25pm **OPENING REMARKS**

Dr Glenn Edwards MBBS MD MAACB FRCPA FACHI
Pathology Director, SA Pathology

5:30pm – 5:45pm **GUEST SPEAKERS**

Associate Professor Susan Branford PhD FFSc (RCPA)
Head, Leukaemia Unit, Genetics and Molecular Pathology
Centre for Cancer Biology *An Alliance between SA Pathology & University of South Australia*

Topic to be presented
"Genomic Advances for Diagnosis and Risk Stratifications in Leukaemia"

5:45pm – 6:00pm

Dr Jovanka King FRACP FRCPA BMBS(Hons) B.Pod DCH(SA)
Immunology, SA Pathology

Topic to be presented
"Newborn screening for primary immunodeficiency diseases"

6:00pm – 6:15pm

Dr Deepak Singhal MBBS, MD, FRACP, FRCPA (Haematology)
Haematology, SA Pathology

Topic to be presented
"Genomic Landscape of Therapy Related Myeloid Neoplasms"

6:15pm – 6:30pm

Professor Angel Lopez AO MBBS PhD FRCPA FAHMS FAA
Director, Centre for Cancer Biology
SA Pathology

Topic to be presented
"How a Pathology-based Discovery led to New Diagnostic and Therapeutic Opportunities"

6:30pm – 7:00pm **PANEL DISCUSSION:** The Future of Research in Public Pathology

Professor Angel Lopez AO MBBS PhD FRCPA FAHMS FAA
Professor Howard Morris PhD FAACB ARCPA
Associate Professor Peter O'Loughlin BSc(hons) PhD FFSc(RCPA
Professor Paddy Phillips MBBS, DPhil, FRACP, MA, FACP, FAHA, FCSANZ
Professor Hamish Scott BSc (Hons), PhD, FFSc (RCPA), FAHMS

7:00pm – 7:45pm **NETWORKING**
Light refreshments provided

SA PATHOLOGY

ascia
australasian society of clinical immunology and allergy inc.

South Australian Branch

Meeting

Tuesday 18th April 2017, 6.15pm start

Martini Restaurant
59A The Parade, Norwood, 5067

Educational Topic

"Newborn screening for primary immunodeficiency diseases"

Dr Jovanka King
Paediatric Immunologist & Immunopathologist

Dinner

Will be served

Sponsored by:

Darryl Hawkey
Territory manager
Stallergens Greer

Please RSVP by 4 pm Friday 14th April
Via "return" email to
Pravin.Hissaria@sa.gov.au

RSVP essential for dinner and early RSVP appreciated

Appendix II: Research Impact

I. The Royal College of Pathologists of Australasia Media Release

II. Media coverage summary

III. Online newspaper article: ‘Doctors want newborns to be screened for ‘bubble boy’ SCID disease’, Sydney Morning Herald online

IV. Printed newspaper article: ‘Newborn screening call: They could have cured it, says mum’, Sydney Morning Herald

The Royal College of Pathologists of Australasia

MEDIA RELEASE **EMBARGOED UNTIL 2 MARCH 2018**

Newborn screening for primary immunodeficiency diseases is a health priority

Dr Jovanka King, a speaker at the Royal College of Pathologists of Australasia's (RCPA) annual conference, 'Pathology Update 2018', will recommend that newborn screening is expanded to include testing for severe immunodeficiency diseases. Dr King will discuss her research on strategies for screening newborn babies for primary immunodeficiency diseases (PID), including Severe Combined Immunodeficiency (SCID), a life-threatening condition where babies are born without a functional immune system.

Dr King, a specialist paediatric immunologist and immunopathologist at SA Pathology at the Women's and Children's Hospital, and the University of Adelaide, explains that this additional screening would significantly improve the outcome for children affected by these disorders, in terms of survival and wellbeing, and anticipates that it would be cost effective.

"Screening babies for primary immune deficiency disorders, including SCID, and evaluating how these tests can be implemented in each state's newborn screening service should be a health priority in Australia. Conditions such as SCID are life-threatening, therefore making a diagnosis and starting treatment as early as possible is essential. Achieving a diagnosis is typically delayed, therefore babies become critically unwell as a result of severe, recurrent infections and other complications. In these cases, they frequently require prolonged hospital and intensive care unit admissions.

"SCID can be cured by a bone marrow or stem cell transplant. There is evidence that if infants with SCID undergo transplantation prior to the age of 3.5 months, their outcomes in terms of survival and long-term health are much improved. Beyond this age, affected babies have a higher burden of infection and other complications due to their untreated disease. The only realistic way to achieve the goal of early transplantation for babies with SCID is to diagnose them early in the newborn period, before they develop symptoms of the disease. This is only achievable through newborn screening.

"The current process for screening newborns in Australia involves each baby having a heel prick blood test when they are between two and three days old. That blood sample is blotted onto a piece of filter paper, and is sent to specialised neonatal screening laboratories for testing. Babies are currently screened for over 40 different conditions, including inborn errors of metabolism and cystic fibrosis, but with recent technological advances we have the potential to expand this further to screen for other important conditions."

Dr King's research has included analysing data from a newborn screening program for PID conducted in Sweden, where almost 60,000 newborn babies were screened for SCID and other forms of PID over a two-year period.

"Our research from Sweden, where I worked previously, has demonstrated that population based newborn screening is an effective way in which to identify babies affected by PID. Affected infants were detected by an abnormal screening test, which enabled rapid medical assessment, confirmatory testing and commencement of treatment within the first weeks of life. This has also been demonstrated in similar studies performed in other countries throughout the world.

"New Zealand recently initiated their screening program for SCID, and almost every state in the United States, and many countries throughout Europe, the Middle East and Asia have initiated

successful screening programs for PID, which have become routine. There is a clear need to establish a newborn screening program for PID in Australia," says Dr King.

In collaboration with other children's hospitals throughout Australia, Dr King will be conducting a cost benefit analysis which is expected to be completed later in the year. Doctor King believes that, not only would this new screening test be beneficial for affected infants and their families around the country, it would also be cost effective.

"The biggest barrier to implementing any new testing strategy is cost, however, economic analyses performed in other countries have demonstrated that it is more cost effective to screen newborns for SCID than it is to manage a critically unwell child in whom diagnosis and treatment was delayed, which is costly to health systems."

For a disease to be included as part of a population-based screening program, it needs to meet certain criteria. Diseases need to be severe, and an effective treatment needs to be available. The testing strategy must also be robust, and have been evaluated on a large scale.

"A retrospective study is underway in our laboratory in South Australia, where we are testing samples from our patients who were diagnosed with PID over the past 17 years. This will determine whether their disease could have been diagnosed in the first weeks of life, and hence if this testing strategy will be effective in our population. Following this, our aim is to secure funding to conduct a prospective screening study, where every baby born in South Australia will undergo screening for these immune deficiency diseases. There are over 300 different kinds of PID and, through our ongoing research efforts, we are also working on new strategies to improve our current capacity to diagnose these severe, life-threatening diseases and improve outcomes for affected patients," explains Dr King.

Dr Jovanka King is a speaker at the RCPA's Pathology Update 2018 conference, 'A Bridge to the Future', which takes place at the ICC Sydney from 2nd until 4th March 2018.

ENDS

About the Royal College of Pathologists of Australasia:

The RCPA is the leading organisation representing pathologists in Australasia. Its mission is to train and support pathologists and to improve the use of pathology testing to achieve better healthcare.

Media enquiries:

Dr Debra Graves
Chief Executive Officer
The Royal College of Pathologists of Australasia
+61 2 8356 5858
Debrag@rcpa.edu.au

Linsey Brown
Managing Director
The Public Relations Agency
+61 425 514 005
Linsey@thepublicrelationsagency.com.au

Media Coverage: Pathology Update 2018 - Newborn Screening

Date	Media Type	Media Outlet	Website URL	Headline	Outlet State
Newborn screening for immunodeficiencies					
6/03/2018 14:11	Online News	dailymail.co.uk	http://www.dailymail.co.uk/wires/aap/article-5466587/Screen-babies-immunodeficiencies.html	Screen babies for immunodeficiencies	National
6/3/2018 5:05:00 PM	Online News	healthtimes.com.au	https://healthtimes.com.au/hub/neonatal/39/news/aap/a-paediatric-specialist-suggests-to-screen-babies-for-immunodeficiencie	A paediatric specialist suggests to screen babies for	National
6/3/2018 2:56:00 PM	Online News	Camden Advertiser	http://www.camdenadvertiser.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:47:00 PM	Online News	The Ararat Advertiser	http://www.araratadvertiser.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:46:00 PM	Online News	Maitland Mercury	http://www.maitlandmercury.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:43:00 PM	Online News	NEWS.com.au	http://www.news.com.au/national/breaking-news/screen-babies-for-immunodeficiencies/news-story/0192d68b3e161854f0684	Screen babies for immunodeficiencies	National
6/3/2018 2:43:00 PM	Online News	Victor Harbor Times	http://www.victorharbortimes.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:42:00 PM	Online News	Murray Valley Standard	http://www.murrayvalleystandard.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:42:00 PM	Online News	Bunbury Mail	http://www.bunburymail.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:40:00 PM	Online News	The Area News	http://www.areanews.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:40:00 PM	Online News	Fairfield Champion	http://www.fairfieldchampion.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:39:00 PM	Online News	Southern Highland News	http://www.southernhighlandnews.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:39:00 PM	Online News	Wimmera Mail Times	http://www.mailtimes.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:36:00 PM	Online News	Liverpool Champion	http://www.liverpoolchampion.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:34:00 PM	Online News	Wollondilly Advertiser	http://www.wollondillyadvertiser.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:34:00 PM	Online News	Manning River Times	http://www.manningrivertimes.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:32:00 PM	Online News	St George & Sutherland Shire Leader	http://www.theleader.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:31:00 PM	Online News	The North West Star	http://www.northweststar.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:31:00 PM	Online News	The Armidale Express	http://www.armidaleexpress.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:31:00 PM	Online News	Daily Liberal	http://www.dailyliberal.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:30:00 PM	Online News	Adelaide Now	http://online.isentialink.com/adelaidenow.com.au/2018/03/06/9df7a2c2-3237-492c-93cb-697c9080b0ab.html	Screen babies for immunodeficiencies	National
6/3/2018 2:30:00 PM	Online News	Brisbane Courier-Mail	http://online.isentialink.com/couriermail.com.au/2018/03/06/094fed08-fc7b-4376-9202-e6c7cfa3bbd1.html	Screen babies for immunodeficiencies	National
6/3/2018 2:30:00 PM	Online News	Geelong Advertiser	http://online.isentialink.com/geelongadvertiser.com.au/2018/03/06/f0e06759-9c37-4869-9f6d-19843e16eb2a.html	Screen babies for immunodeficiencies	National
6/3/2018 2:30:00 PM	Online News	The Australian	http://online.isentialink.com/theaustralian.com.au/2018/03/06/29a4b782-30ab-4a6d-a573-7dbb26217635.html	Screen babies for immunodeficiencies	National
6/3/2018 2:30:00 PM	Online News	Herald Sun	http://online.isentialink.com/heraldsun.com.au/2018/03/06/c089f081-216a-4a20-83da-5f2e075a1051.html	Screen babies for immunodeficiencies	National
6/3/2018 2:30:00 PM	Online News	Gold Coast Bulletin	http://online.isentialink.com/goldcoastbulletin.com.au/2018/03/06/295642ad-572c-47e5-ad2d-115983792630.html	Screen babies for immunodeficiencies	National
6/3/2018 2:30:00 PM	Online News	Northern Territory News	http://online.isentialink.com/ntnews.com.au/2018/03/06/95407ba0-40e5-4944-81c5-b7a0d7db0b13.html	Screen babies for immunodeficiencies	National
6/3/2018 2:30:00 PM	Online News	Daily Telegraph Australia	http://online.isentialink.com/dailytelegraph.com.au/2018/03/06/f9a7849a-0ca6-431c-8477-43b9ca4556c2.html	Screen babies for immunodeficiencies	National
6/3/2018 2:30:00 PM	Online News	The Mercury	http://online.isentialink.com/themercury.com.au/2018/03/06/2f7fc6be-e1bf-40fa-ab02-aee8f3c1b2d0.html	Screen babies for immunodeficiencies	National
6/3/2018 2:30:00 PM	Online News	Weekly Times Now	http://online.isentialink.com/weeklytimesnow.com.au/2018/03/06/7ad746b1-36ec-43e8-87af-8bc5ec49b6b1.html	Screen babies for immunodeficiencies	National
6/3/2018 2:30:00 PM	Online News	Townsville Bulletin	http://online.isentialink.com/townsvillebulletin.com.au/2018/03/06/549c225e-f64d-4a25-a38d-978c0eb68fd2.html	Screen babies for immunodeficiencies	National
6/3/2018 2:30:00 PM	Online News	Cairns Post	http://online.isentialink.com/cairnspost.com.au/2018/03/06/03626710-beeb-4a80-bd58-3312b0ccd73f.html	Screen babies for immunodeficiencies	National
6/3/2018 2:29:00 PM	Online News	Central Western Daily	http://www.centralwesterndaily.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:28:00 PM	Online News	Bega District News	http://www.begadistrictnews.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:28:00 PM	Online News	Newcastle Herald	http://www.theherald.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:28:00 PM	Online News	Mandurah Mail	http://www.mandurahmail.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:28:00 PM	Online News	The Border Mail	http://www.bordermail.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:26:00 PM	Online News	Mudgee Guardian	http://www.mudgeeguardian.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:25:00 PM	Online News	Bathurst Western Advocate	http://www.westernadvocate.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:25:00 PM	Online News	Illawarra Mercury	http://www.illawarramercury.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/03/2018 14:24	Online News	Bendigo Advertiser	http://www.bendigoadvertiser.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:24:00 PM	Online News	Tasmanian Examiner	http://www.examiner.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:24:00 PM	Online News	Wagga Wagga Daily Advertiser	http://www.dailyadvertiser.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:23:00 PM	Online News	Macarthur Advertiser	http://www.macarthuradvertiser.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:23:00 PM	Online News	Blue Mountains Gazette	http://www.bluemountainsgazette.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:22:00 PM	Online News	Hawkesbury Gazette	http://www.hawkesburygazette.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:22:00 PM	Online News	Port Macquarie News	http://www.portnews.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:22:00 PM	Online News	South Coast Register	http://www.southcoastregister.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:22:00 PM	Online News	Northern Daily Leader	http://www.northerndailyleader.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:22:00 PM	Online News	Port Lincoln Times	http://www.portlincolntimes.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:21:00 PM	Online News	Ballarat Courier	http://www.thecourier.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:20:00 PM	Online News	Lithgow Mercury	http://www.lithgowmercury.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:20:00 PM	Online News	Goulburn Post	http://www.goulburnpost.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:20:00 PM	Online News	redlandcitybulletin.com.au	http://www.redlandcitybulletin.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:18:00 PM	Online News	The Advocate	http://www.theadvocate.com.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:16:00 PM	Online News	World News Australia	https://www.sbs.com.au/news/screen-babies-for-immunodeficiencies	Screen babies for immunodeficiencies	National
6/3/2018 2:15:00 PM	Online News	Yahoo! New Zealand	https://nz.news.yahoo.com/screen-babies-immunodeficiencies-030820336--spt.html	Screen babies for immunodeficiencies	National
6/3/2018 2:15:00 PM	Online News	The Stawell Times-News	http://www.stawelltimes.com.au/story/5268010/screen-babies-for-immunodeficiencies/?cs=7	Screen babies for immunodeficiencies	National
6/3/2018 2:12:00 PM	Online News	Warrnambool Standard	http://www.standard.net.au/story/5268010/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:11:00 PM	Online News	Yahoo! News Australia	https://au.news.yahoo.com/a/39418049/screen-babies-for-immunodeficiencies/	Screen babies for immunodeficiencies	National
6/3/2018 2:10:00 PM	Online News	centralnews.com.au	http://www.centralnews.com.au/@breaking-news/2018/03/06/94615/screen-babies-for-immunodeficiencies	Screen babies for immunodeficiencies	National
5/3/2018 1:33:00 PM	Online News	Essential Baby	http://www.essentialbaby.com.au/baby/baby-health/doctors-want-newborns-to-be-screened-for-bubble-boy-scid-disease-2018	Doctors want newborns to be screened for 'bubble	National

2/3/2018 12:09:00 AM	Online News	WA Today	http://www.watoday.com.au/national/doctors-want-newborns-to-be-screened-for-bubble-boy-scid-disease-20180301-p4z2bc.h	Doctors want newborns to be screened for 'bubble	National
2/3/2018 12:04:00 AM	Online News	Canberra Times	http://www.canberratimes.com.au/national/doctors-want-newborns-to-be-screened-for-bubble-boy-scid-disease-20180301-p4z	Doctors want newborns to be screened for 'bubble	National
2/3/2018 12:04:00 AM	Online News	Brisbane Times	https://www.brisbanetimes.com.au/healthcare/doctors-want-newborns-to-be-screened-for-bubble-boy-scid-disease-20180301-	Doctors want newborns to be screened for 'bubble	National
2/3/2018 12:02:00 AM	Online News	Sydney Morning Herald	https://www.smh.com.au/healthcare/doctors-want-newborns-to-be-screened-for-bubble-boy-scid-disease-20180301-p4z2bc.htı	Doctors want newborns to be screened for 'bubble	National
2/3/2018 12:01:00 AM	Online News	The Age	https://www.theage.com.au/healthcare/doctors-want-newborns-to-be-screened-for-bubble-boy-scid-disease-20180301-p4z2bc	Doctors want newborns to be screened for 'bubble	National
2/3/2018 12:00:00 AM	Newspaper	Sydney Morning Herald	http://www.mediaportal.com/0zo1099928005	They could have cured it, says mum	NSW
1/3/2018 9:25:00 PM	Online News	Sydney Morning Herald	http://www.isentialink.com/?u=smh.com.au%2fhealthcare%2fdoctors-want-newborns-to-be-screened-for-bubble-boy-scid-disez	Doctors want newborns to be screened for 'b	National
1/3/2018 12:00:00 AM	Trade Magazine	Retail Pharmacy	http://www.mediaportal.com/0ig1099928008	Pregnant pause	National
2/3/2018 1:06:00 PM	Online News	New Zealand Doctor Online	https://www.nzdoctor.co.nz/article/undoctored/newborn-screening-primary-immunodeficiency-diseases-health-priority	Newborn screening for primary immunodeficiency c	National
2/3/2018 5:32:01 PM	AM Radio	2EC	NA	There are calls to expand screening of newborns for	NSW
2/3/2018 12:06:03 PM	FM Radio	7LA	NA	The Royal College of Pathologists has called to expa	TAS
2/3/2018 12:02:26 PM	FM Radio	WAVE FM	NA	There are calls to expand screening of newborns for	NSW
2/3/2018 10:34:31 AM	AM Radio	2EC	NA	There are calls to expand screening of newborns for	NSW
2/3/2018 9:02:22 AM	AM Radio	4BC	NA	The Royal College of Pathologists is calling for an ex	QLD
2/3/2018 9:00:58 AM	FM Radio	98.9FM	NA	There are calls for screening expansion on infants fc	QLD
2/3/2018 8:29:43 AM	AM Radio	2EC	NA	There are calls to expand screening of newborns for	NSW
2/3/2018 8:09:07 AM	AM Radio	3AW	NA	Interview with Dr Jovanka King, paediatric immunol	VIC
2/3/2018 7:59:56 AM	FM Radio	POWER FM Illawarra	NA	There are calls for more screening tests for babies a	NSW
2/3/2018 7:33:06 AM	FM Radio	Hot FM 91.1	NA	The Royal College of Pathologists of Australasia's Dr	QLD
2/3/2018 7:32:30 AM	AM Radio	2GB	NA	There are calls for more screening tests for babies a	NSW
2/3/2018 7:03:25 AM	FM Radio	Curtin FM	NA	There are calls for more screening tests for babies a	WA
2/3/2018 6:32:43 AM	AM Radio	4BC	NA	There have been calls to fund new-born screening p	QLD
2/3/2018 6:31:53 AM	AM Radio	2EC	NA	There are calls to expand screening of newborns for	NSW
2/3/2018 6:03:05 AM	FM Radio	Curtin FM	NA	There are calls to fund newborn screening program:	WA
2/3/2018 5:32:38 AMA45:	AM Radio	2GB	NA	There have been calls for greater funding of newbo	NSW

Radio Release Report - Newborn Screening

Download Source	Grab	Broadcast Stations & Syndicates	Potential Audience	Average Audience
Kingaroy - Crow FM	1	Kingaroy & Lower Burnett (Qld) - Crow FM	N/A	N/A
Kingaroy - Crow FM	6	Kingaroy & Lower Burnett (Qld) - Crow FM	N/A	N/A
Parkes - 2PK/ROK	6	Parkes (NSW) - 2PK	28,200	1,400
		Parkes (NSW) - Rok FM	28,200	1,500
National - AIR National News	4	Adelaide (SA) - PBA FM	N/A	N/A
		Albany (WA) - Gold MX	N/A	N/A
		Albury/Wodonga (NSW/Vic) - 2REM FM 107.3	N/A	N/A
		Augusta (WA) - 2 Oceans FM	N/A	N/A
		Bacchus Marsh (Vic) - Apple FM	N/A	N/A
		Bathurst (NSW) - Life FM	N/A	N/A
		Beaudesert (Qld) - Beau FM 101.5	N/A	N/A
		Boonah (Qld) - Rim FM	N/A	N/A
		Bordertown (SA) - 5TCB	N/A	N/A
		Bribie Island (Qld) - 88.0 FM	N/A	N/A
		Brisbane (Qld) - Bay FM 100.3	N/A	N/A
		Bunbury (WA) - 103.7 FM	N/A	N/A
		Byron Bay (NSW) - Bay FM	N/A	N/A
		Cairns (Qld) - Cairns FM 89.1	N/A	N/A
		Canberra & Tuggeranong (ACT) - Artsound FM	N/A	N/A
		Carnarvon & Geraldton (WA) - Radio Mama	N/A	N/A
		Cessnock (NSW) - 2CHR FM	N/A	N/A
		Collie (WA) - 1089 AM	N/A	N/A
		Coraki (NSW) - Richmond Valley Radio	N/A	N/A
		Darwin (NT) - 97 Seven	N/A	N/A
		Devonport (Tas) - 7RPH 96.1 FM	N/A	N/A
		Dubbo (NSW) - 88.9 DC FM	N/A	N/A
		Dungog (NSW) - FM 107.9	N/A	N/A
		Echuca/Moama (Vic/NSW) - EM FM 104.7	N/A	N/A
		Esperance (WA) - Hope FM	N/A	N/A
		Fraser Coast (Qld) - Fraser Coast FM 107.5	N/A	N/A
		Gippsland & Phillip Island (Vic) - 3MFM	N/A	N/A
		Gold Coast (Qld) - 94.1 FM	586,000	N/A
		Goolwa (SA) - Alex FM 96.3	N/A	N/A
		Gosford (NSW) - 94ONE	N/A	N/A
		Goulburn (NSW) - FM 103.3	N/A	N/A
		Grafton (NSW) - Big Rig Radio	N/A	N/A
		Healesville (Vic) - Flow FM	N/A	N/A
		Hepburn Springs (Vic) - Hepburn Community R	N/A	N/A
		Hobart (Tas) - 7RPH 864 AM	N/A	N/A

		Hobart (Tas) - 99.3 Edge FM	N/A	N/A
		Holbrook (NSW) - 2GHR FM	N/A	N/A
		Inverell (NSW) - STA FM	N/A	N/A
		Kangaroo Island (SA) - Kix FM 90.7	N/A	N/A
		Katherine (NT) - 8KTR	N/A	N/A
		Kingaroy & Lower Burnett (Qld) - Crow FM	N/A	N/A
		Lake Macquarie (NSW) - Radio Yesteryear 97.	N/A	N/A
		Lameroo (SA) - 3MBR FM 107.5	N/A	N/A
		Launceston (Tas) - 7RPH 106.9 FM	N/A	N/A
		Launceston (Tas) - Way FM	N/A	N/A
		Lightning Ridge (NSW) - Opal FM	N/A	N/A
		Loxton & Riverland (SA) - Life FM	N/A	N/A
		Mackay (Qld) - 4CRM 107.5 FM	N/A	N/A
		Mackay, Sarina & Whitsundays (Qld) - My 105.	N/A	N/A
		Mansfield (Vic) - Radio Mansfield 99.7	N/A	N/A
		Maryborough (Vic) - Goldfields FM	N/A	N/A
		Marysville (Vic) - Flow FM	N/A	N/A
		Melbourne (Vic) - 3SER 97.7 FM	N/A	N/A
		Melbourne (Vic) - 3WBC 94.1 FM	N/A	N/A
		Melbourne (Vic) - Plenty Valley FM 88.6	N/A	N/A
		Moranbah (Qld) - 4RFM 96.9	N/A	N/A
		Morwell (Vic) - Gippsland FM	N/A	N/A
		Mt Gambier (SA) - Lime FM	42,400	N/A
		Mt Isa (Qld) - Rhema FM 107.5	N/A	N/A
		Murrayville (Vic) - 3MBR FM 103.5	N/A	N/A
		Murrindindi/Marysville (Vic) - UGFM	N/A	N/A
		Narrandera (NSW) - Spirit FM	N/A	N/A
		Newcastle (NSW) - Radio 1629 AM	N/A	N/A
		Newman (WA) - 6NEW	N/A	N/A
		Nhulunbuy (NT) - Gove FM 106.9	N/A	N/A
		Orange (NSW) - FM 107.5	N/A	N/A
		Port Hedland (WA) - 101.3 FM	N/A	N/A
		Port Pirie (SA) - Trax FM 105.1	N/A	N/A
		Port Stephens (NSW) - Port Stephens FM	N/A	N/A
		Qld/NT/SA/Tas - RadioTAB (91 locations)	N/A	N/A
		Regional SA - Flow FM (15 locations)	104,800	10,500
		Roxby Downs (SA) - Rox FM	N/A	N/A
		Seymour (Vic) - Seymour FM	N/A	N/A
		Shoalhaven Coast (NSW) - Triple U FM	N/A	N/A
		Southern Highlands (NSW) - Sounds of the Mo	N/A	N/A
		Stanthorpe (Qld) - Ten FM	N/A	N/A
		Sunshine Coast (Qld) - Sunshine FM	355,000	N/A
		Sydney (NSW) - Sky Sports Radio	4,360,000	5,000
		Taree (NSW) - 2TLP 103.3 FM	N/A	N/A
		Tasmania - Coast FM (4 Locations)	N/A	N/A
		Tasmania - Star FM (3 Locations)	N/A	N/A
		Temora (NSW) - Tem FM	N/A	N/A
		Tennant Creek (NT) - Flow FM	N/A	N/A
		Tenterfield/Stanthorpe (NSW/Qld) - Ten FM	N/A	N/A
		Townsville (Qld) - Triple T	163,300	N/A
		Tumbarumba (NSW) - Radio Upper Murray	N/A	N/A
		Wagga Wagga (NSW) - Life FM	N/A	N/A
		Walwa (Vic) - Radio Upper Murray	N/A	N/A
		Warwick (Qld) - Rainbow FM	N/A	N/A
		Whyalla (SA) - 5YYY	N/A	N/A
		Yarra Junction (Vic) - 99.1 Yarra Valley FM	N/A	N/A
		Yarraman (Qld) - 4WHO 99.7 FM	N/A	N/A
National - AIR National News	1	Adelaide (SA) - PBA FM	N/A	N/A
		Albany (WA) - Gold MX	N/A	N/A
		Albury/Wodonga (NSW/Vic) - 2REM FM 107.3	N/A	N/A
		Augusta (WA) - 2 Oceans FM	N/A	N/A
		Bacchus Marsh (Vic) - Apple FM	N/A	N/A
		Bathurst (NSW) - Life FM	N/A	N/A
		Beaudesert (Qld) - Beau FM 101.5	N/A	N/A
		Boonah (Qld) - Rim FM	N/A	N/A
		Bordertown (SA) - 5TCB	N/A	N/A
		Bribie Island (Qld) - 88.0 FM	N/A	N/A
		Brisbane (Qld) - Bay FM 100.3	N/A	N/A
		Bunbury (WA) - 103.7 FM	N/A	N/A
		Byron Bay (NSW) - Bay FM	N/A	N/A
		Cairns (Qld) - Cairns FM 89.1	N/A	N/A
		Canberra & Tuggeranong (ACT) - Artsound FM	N/A	N/A

	Carnarvon & Geraldton (WA) - Radio Mama	N/A	N/A
	Cessnock (NSW) - 2CHR FM	N/A	N/A
	Collie (WA) - 1089 AM	N/A	N/A
	Coraki (NSW) - Richmond Valley Radio	N/A	N/A
	Darwin (NT) - 97 Seven	N/A	N/A
	Devonport (Tas) - 7RPH 96.1 FM	N/A	N/A
	Dubbo (NSW) - 88.9 DC FM	N/A	N/A
	Dungog (NSW) - FM 107.9	N/A	N/A
	Echuca/Moama (Vic/NSW) - EM FM 104.7	N/A	N/A
	Esperance (WA) - Hope FM	N/A	N/A
	Fraser Coast (Qld) - Fraser Coast FM 107.5	N/A	N/A
	Gippsland & Phillip Island (Vic) - 3MFM	N/A	N/A
	Gold Coast (Qld) - 94.1 FM	586,000	N/A
	Goolwa (SA) - Alex FM 96.3	N/A	N/A
	Gosford (NSW) - 94ONE	N/A	N/A
	Goulburn (NSW) - FM 103.3	N/A	N/A
	Grafton (NSW) - Big Rig Radio	N/A	N/A
	Healesville (Vic) - Flow FM	N/A	N/A
	Hepburn Springs (Vic) - Hepburn Community R	N/A	N/A
	Hobart (Tas) - 7RPH 864 AM	N/A	N/A
	Hobart (Tas) - 99.3 Edge FM	N/A	N/A
	Holbrook (NSW) - 2GHR FM	N/A	N/A
	Inverell (NSW) - STA FM	N/A	N/A
	Kangaroo Island (SA) - Kix FM 90.7	N/A	N/A
	Katherine (NT) - 8KTR	N/A	N/A
	Kingaroy & Lower Burnett (Qld) - Crow FM	N/A	N/A
	Lake Macquarie (NSW) - Radio Yesteryear 97.	N/A	N/A
	Lameroo (SA) - 3MBR FM 107.5	N/A	N/A
	Launceston (Tas) - 7RPH 106.9 FM	N/A	N/A
	Launceston (Tas) - Way FM	N/A	N/A
	Lightning Ridge (NSW) - Opal FM	N/A	N/A
	Loxton & Riverland (SA) - Life FM	N/A	N/A
	Mackay (Qld) - 4CRM 107.5 FM	N/A	N/A
	Mackay, Sarina & Whitsundays (Qld) - My 105.	N/A	N/A
	Mansfield (Vic) - Radio Mansfield 99.7	N/A	N/A
	Maryborough (Vic) - Goldfields FM	N/A	N/A
	Marysville (Vic) - Flow FM	N/A	N/A
	Melbourne (Vic) - 3SER 97.7 FM	N/A	N/A
	Melbourne (Vic) - 3WBC 94.1 FM	N/A	N/A
	Melbourne (Vic) - Plenty Valley FM 88.6	N/A	N/A
	Moranbah (Qld) - 4RFM 96.9	N/A	N/A
	Morwell (Vic) - Gippsland FM	N/A	N/A
	Mt Gambier (SA) - Lime FM	42,400	N/A
	Mt Isa (Qld) - Rhema FM 107.5	N/A	N/A
	Murrayville (Vic) - 3MBR FM 103.5	N/A	N/A
	Murrindindi/Marysville (Vic) - UGFM	N/A	N/A
	Narrandera (NSW) - Spirit FM	N/A	N/A
	Newcastle (NSW) - Radio 1629 AM	N/A	N/A
	Newman (WA) - 6NEW	N/A	N/A
	Nhulunbuy (NT) - Gove FM 106.9	N/A	N/A
	Orange (NSW) - FM 107.5	N/A	N/A
	Port Hedland (WA) - 101.3 FM	N/A	N/A
	Port Pirie (SA) - Trax FM 105.1	N/A	N/A
	Port Stephens (NSW) - Port Stephens FM	N/A	N/A
	Qld/NT/SA/Tas - RadioTAB (91 locations)	N/A	N/A
	Regional SA - Flow FM (15 locations)	104,800	10,500
	Roxby Downs (SA) - Rox FM	N/A	N/A
	Seymour (Vic) - Seymour FM	N/A	N/A
	Shoalhaven Coast (NSW) - Triple U FM	N/A	N/A
	Southern Highlands (NSW) - Sounds of the Mo	N/A	N/A
	Stanthorpe (Qld) - Ten FM	N/A	N/A
	Sunshine Coast (Qld) - Sunshine FM	355,000	N/A
	Sydney (NSW) - Sky Sports Radio	4,360,000	5,000
	Taree (NSW) - 2TLP 103.3 FM	N/A	N/A
	Tasmania - Coast FM (4 Locations)	N/A	N/A
	Tasmania - Star FM (3 Locations)	N/A	N/A
	Temora (NSW) - Tem FM	N/A	N/A
	Tennant Creek (NT) - Flow FM	N/A	N/A
	Tenterfield/Stanthorpe (NSW/Qld) - Ten FM	N/A	N/A
	Townsville (Qld) - Triple T	163,300	N/A
	Tumbarumba (NSW) - Radio Upper Murray	N/A	N/A
	Wagga Wagga (NSW) - Life FM	N/A	N/A

		Walwa (Vic) - Radio Upper Murray	N/A	N/A
		Warwick (Qld) - Rainbow FM	N/A	N/A
		Whyalla (SA) - 5YYY	N/A	N/A
		Yarra Junction (Vic) - 99.1 Yarra Valley FM	N/A	N/A
		Yarraman (Qld) - 4WHO 99.7 FM	N/A	N/A
Innisfail/Mareeba - Local	6	Innisfail (Qld) - 4AY	25,000	N/A
02/03/2018 - 12:34		Innisfail (Qld) - 4KZ	25,000	1,200
		Innisfail (Qld) - Kool FM	25,000	1,400
		Mareeba (Qld) - 4AM	39,600	1,900
		Mareeba (Qld) - Kik FM	N/A	N/A
Wollongong - Wave	6	Wollongong (NSW) - Wave FM	230,500	7,000
02/03/2018 - 11:02				
New England - Local	6	Armidale (NSW) - 2AD	28,900	1,400
02/03/2018 - 10:15		Armidale (NSW) - FM 100.3	28,900	1,600
		Gunnedah (NSW) - 2MO	36,700	1,800
		Gunnedah (NSW) - Triple G	36,700	2,000
		Tamworth (NSW) - 2TM	53,100	2,600
		Tamworth (NSW) - One FM	53,100	2,900
National - NIRS National New	5	National - NIRS Affiliates (125 Locations)	N/A	N/A
02/03/2018 - 08:06				
National - NIRS National New	4	National - NIRS Affiliates (125 Locations)	N/A	N/A
02/03/2018 - 08:06				
National - NIRS National New	3	National - NIRS Affiliates (125 Locations)	N/A	N/A
02/03/2018 - 08:06				
National - NIRS National New	2	National - NIRS Affiliates (125 Locations)	N/A	N/A
02/03/2018 - 08:06				
National - NIRS National New	1	National - NIRS Affiliates (125 Locations)	N/A	N/A
02/03/2018 - 08:06				
Gippsland (Cooma) - Capital I	4	Cooma (NSW) - 2XL	17,700	900
02/03/2018 - 06:55		Cooma (NSW) - Snow FM	17,700	1,000
		Warragul & Gippsland (Vic) - 3GG	177,800	2,700
Gippsland (Cooma) - Capital I	6	Cooma (NSW) - 2XL	17,700	900
02/03/2018 - 06:51		Cooma (NSW) - Snow FM	17,700	1,000
		Warragul & Gippsland (Vic) - 3GG	177,800	2,700
Innisfail - 4KZ Breakfast (Johr	6	Innisfail (Qld) - 4KZ	25,000	1,200
02/03/2018 - 06:50				
Innisfail - 4KZ Breakfast (Johr	5	Innisfail (Qld) - 4KZ	25,000	1,200
02/03/2018 - 06:50				
Innisfail - 4KZ Breakfast (Johr	4	Innisfail (Qld) - 4KZ	25,000	1,200
02/03/2018 - 06:50				
Innisfail - 4KZ Breakfast (Johr	3	Innisfail (Qld) - 4KZ	25,000	1,200
02/03/2018 - 06:50				
Innisfail - 4KZ Breakfast (Johr	2	Innisfail (Qld) - 4KZ	25,000	1,200
02/03/2018 - 06:50				
Innisfail - 4KZ Breakfast (Johr	1	Innisfail (Qld) - 4KZ	25,000	1,200
02/03/2018 - 06:50				
Mid North Coast NSW - Local	6	Coffs Harbour (NSW) - 2HC 639 AM	63,100	1,200
02/03/2018 - 06:41		Grafton (NSW) - 2GF	42,800	2,100
		Grafton (NSW) - FM 104.7	42,800	2,300
Ballarat - Voice FM	6	Ballarat (Vic) - Voice FM	N/A	N/A
02/03/2018 - 05:50				
Bendigo & Castlemaine - KLF	6	Bendigo & Castlemaine (Vic) - KLFM	N/A	N/A
02/03/2018 - 05:50				
Nowra - 2ST/Power	6	Nowra, Bowral & Ulladulla (NSW) - 2ST	127,700	6,300
02/03/2018 - 05:45		Nowra, Bowral & Ulladulla (NSW) - Power FM	127,700	6,900
Albury/Wodonga - 2AY	6	Albury/Wodonga (NSW/Vic) - 2AY	107,000	3,000
02/03/2018 - 05:33		Regional Victoria - Ace Radio Affiliates*	N/A	N/A
Bega - 2EC/Power	6	Bega (NSW) - 2EC	59,100	2,900
02/03/2018 - 05:01		Bega (NSW) - Power FM	59,100	3,200

NATIONAL HEALTHCARE

Doctors want newborns to be screened for 'bubble boy' SCID disease

By **Esther Han**
1 March 2018 — 9:25pm

In the first five weeks of his life, Harrison Draper went from being a healthy, pink-skinned newborn to a sick child who left doctors stumped.

He stopped gaining weight and developed a painful abscess on his bottom that wouldn't heal.

"The doctors kept saying, 'It's OK, it happens,' " his mother Tracey Brown, from Orange, said. "But I kept telling them, 'Stop saying that, my gut is screaming at me that something's wrong', but he didn't have other symptoms."

In numbers

3.5 months
Babies that undergo transplantation before 3.5 months have a greater chance of survival and recovery.

58,000
SCID affects one in 58,000 births internationally.

40
Babies are currently screened for more than 40 different conditions, including inborn errors of metabolism and cystic fibrosis.

Harrison Draper, pictured with his mother Tracey, has severe combined immunodeficiency, a life-threatening condition.

Photo: Peter Rae

At 11 weeks, Harrison was struck down by a double blow of meningitis and pneumonia. His doctors, confused, conducted "invasive" tests and ordered the pivotal blood test that provided the answer: Harrison had severe combined immunodeficiency (SCID) which meant he had virtually no immune protection from bacteria and viruses.

"What saddens me is that if they had found out sooner, his prognosis would've been incredibly different, they could've cured it," she said of Harrison, who will turn 11 in four days.

"He has damage because he got so sick before he was diagnosed; he has neurological damage, hearing loss, an intellectual delay, things that will affect the rest of his life."

Harrison Draper recovering in hospital after a bone marrow transplant.

Doctors are calling for newborn screening to be expanded to include testing for severe forms of primary immunodeficiency diseases, in particular SCID, which, if detected early enough, can be cured by a bone marrow or stem cell transplant.

SCID, commonly known as the "bubble boy" disease, is a severe and life-threatening disease that affects one in 58,000 births internationally.

At the Royal College of Pathologists of Australasia's annual conference Pathology Update on Friday, paediatric immunologist Dr Jovanka King said testing for SCID would significantly improve or even save the lives of affected children.

"The problem is affected babies first appear to be healthy and it's not until they develop lots of infections that they're diagnosed and by then there have been lots of complications," she said.

"If we can do the transplant before they're 3½ months, they fare a lot better in terms of survival and have fewer long-term complications."

At present, newborn babies are screened for SCID in New Zealand, most states in the US and many countries throughout Europe, the Middle East and Asia.

Dr King, in collaboration with several children's hospitals, is conducting a cost-benefit analysis, which she expects to complete by the end of the year.

"Economic analyses performed in other countries have demonstrated it is more cost effective to screen newborns for SCID than it is to manage a critically unwell child in whom diagnosis and treatment was delayed," she said.

Dr Melanie Wong, a paediatric immunologist at Westmead Children's Hospital, said her hospital saw about one case a year, but it was one too many.

She said some newborns were screened for SCID based on family history. Babies diagnosed early have a 90 per cent survival rate, compared to those diagnosed late, who have a 40 per cent survival rate.

Harrison with his older sister Ella, who was the perfect donor match.

"Some come at two to three months with devastating infections and need intensive care and ventilation and there are ones who present at six to eight months because of their failure to thrive," she said.

"Then there are a few who have more insidious presentations and, when you go to transplant, they've picked up a whole lot of viruses and that makes it more difficult to do a transplant."

Christine Jeffery, from the Immune Deficiencies Foundation, which counts 14 surviving SCID patients as members, strongly supported the call, saying most forms of primary immunodeficiency had no cure, but SCID did.

"SCID fulfils all the internationally recognised criteria for a clinical condition to be screened for at birth through newborn screening using the standard Guthrie [dried blood spot] sample," its position statement reads.

On their way to a surgery at Westmead Children's Hospital, Ms Brown said all newborns should be screened for SCID.

Harrison has had several bone marrow transplants – at four months, 18 months and eight years – as well as chemotherapy.

"For us, my daughter Ella is the perfect match, so we were extremely lucky to find a match so quickly," she said.

"There are kids overseas who are diagnosed at birth but have the transplant three months later because they couldn't find a donor match."

The federal Health Department said it was up to each of the states and territories to determine which conditions would be screened for by their program.

Dr Jovanka King, a specialist paediatric immunologist and immunopathologist at SA Pathology at the Women's and Children's Hospital and the University of Adelaide.

Photo:

HEALTHCARE BABY HEALTH PREGNANCY

Esther Han

Esther Han is a health reporter at The Sydney Morning Herald. She has previously been

NEWBORN SCREENING CALL

They could have cured it, says mum

Esther Han
Health reporter

In the first five weeks of his life, Harrison Draper went from being a healthy, pink-skinned newborn to a sick child who left doctors stumped.

He stopped gaining weight and developed a painful abscess on his bottom that wouldn't heal.

"The doctors kept saying, 'It's OK, it happens'," his mother Tracey Brown, from Orange, said. "But I kept telling them, 'stop saying that, my gut is screaming at me that something's wrong', but he didn't have other symptoms."

At 11 weeks, Harrison was struck down by a double blow of meningitis and pneumonia.

His doctors, confused, conducted "invasive" tests and ordered the pivotal blood test that provided the answer: Harrison had severe combined immunodeficiency (SCID), which meant he had virtually no immune protection from bacteria and viruses.

"What saddens me is that if they had found out sooner, his prognosis would be incredibly different, they could've cured it," she said of Harrison, who turns 11 this week.

"He has damage because he got so sick before he was diagnosed; he has neurological damage, hearing loss, an intellectual delay, things that will affect the rest of his life."

'Bubble boy': Tracey Brown and her son Harrison, who was just about to go in for eye surgery. Photo: Peter Rae

Doctors are calling for newborn screening to be expanded to include testing for severe forms of primary immunodeficiency diseases, primarily SCID, which, if detected early enough, can be cured by a bone marrow or stem cell transplant. SCID, commonly known as the "bubble boy" disease, is a severe and life-threatening disease that affects one in 58,000 births internationally.

At the Royal College of Pathologists of Australasia's (RCPA) conference Pathology Update today, paediatric immunologist Dr Jovanka King will say in her address, testing for SCID would significantly improve or even save the lives of affected children. "The problem is affected babies first appear to be healthy and it's not until they develop lots of infections that they're diagnosed and by then there have been lots of complications," she will say. "If we can do the transplant before they're 3½ months, they fare a lot better in terms of survival and have fewer long-term complications."

At present, newborn babies are screened for SCID in New Zealand, most states in the US and many countries throughout Europe, the Middle East and Asia.

Dr King, in collaboration with several children's hospitals, is conducting a cost-benefit analysis, which she expects will be completed by the end of the year.

"The biggest barrier is cost however economic analyses performed in other countries have shown it is more cost effective to screen newborns for SCID than it is to manage a critically unwell child in whom diagnosis and treatment was delayed," she said.

Dr Melanie Wong, a paediatric immunologist at Westmead Children's Hospital, said her hospital saw about one case a year, but it was one too many. She said some newborns were screened for SCID based on family history. Babies diagnosed early have a 90 per cent survival rate, compared to those diagnosed late, who have a 40 per cent survival rate.

Christine Jeffery, from the Immune Deficiencies Foundation, strongly supported the call, saying most forms of primary immunodeficiency had no cure, but SCID did.

Appendix III: Additional Publications

I. Chan, D., **King, J**., Dzeladini, L., Marchand, B., Gold, M., Quinn, P. (2017) Ruxolitinib use in a child with chronic mucocutaenous candidiasis and autosomal dominant signal transducer and activator of transcription 1 gain of function (STAT1 GOF) mutation. Intern Med J, 47: 28. doi:10.1111/imj.78_13578

II. Gold, M., Chan, D., Marchand, B., Dzeladini, L., **King, J**., Cheung, A., Quinn, P. (2017) Intussusception: A Novel presentation of activated phosphoinositide 3-kinase-Δ (PI3KΔ) syndrome (APDS). Intern Med J, 47: 28–29. doi:10.1111/imj.80_13578

III. Quinn, P., Pearson, C., Cheung, A., **King, J**., Chan, D., Marchand, B., Rudaks, D., Quach, A., Ferrante, A., Gold, M. (2017) Combined immunodeficiency due to deficiency of actin-related protein complex 1B (ARPC1B). Intern Med J, 47: 29–30. doi:10.1111/imj.84_13578

IV. Small A., **King J.**, Rathjen D., Ferrante A. 'The Role of Phagocytes in Immunity to *Candida albicans*', Chapter in *Candida Albicans,* IntechOpen, UK, 2018, ISBN 978-953-51-6399-2

P77
STRENGTHENING ACCESS CRITERIA FOR IMMUNOGLOBULIN IN AUSTRALIA

J Roberts,[1] J Cameron,[1] L Wall,[1] N Wicks,[1] P Hetzel[2] and M Stone[1]

[1]National Blood Authority, Canberra, Australia
[2]Consultant to the National Blood Authority, Canberra, Australia

The National Blood Authority is standardising and strengthening access criteria for immunoglobulin (Ig) funded by all Australian governments under the national blood arrangements.

The '*Criteria for the Clinical Use of Intravenous Immunoglobulin in Australia*' (the *Criteria*) was first published in 2007 and updated in 2012. After an independent review of the governance and access arrangements, the National Blood Authority (NBA) established a network of governance committees including specialist working groups (SWGs) and developed an online authorisation system (BloodSTAR). The Criteria currently included in BloodSTAR reflects the 2012 second edition of the *Criteria*. All states and territories, with the exception of NSW, now use BloodSTAR.

The SWGs reviewed the evidence and made revisions for V3 of *the Criteria*. In 2015, public consultation was held on proposed V3 changes for conditions where Ig has an Established or Emerging Therapeutic Role. In 2017, public consultation was held on proposed V3 changes for conditions listed for Use in Exceptional Circumstances Only and for indications where Ig use is Not Supported.

The V3 changes were endorsed by the National Immunoglobulin Governance Advisory Committee prior to presentation to the Jurisdictional Blood Committee for approval.

The proposed changes, which aim to more clearly articulate and standardise the diagnostic, qualifying and review criteria, are required to assist with managing the growth in demand for this precious, human-derived product by ensuring it is only used for clinically appropriate purposes. The revised *Criteria* will ensure strengthened qualifying and review criteria, initial and continuing authorisation periods', dosing controls and ensure the submission of supporting evidence.

The *V3 Criteria* will undergo an extensive process to upload into BloodSTAR and transition existing patients to the new criteria.

P78
RUXOLITINIB USE IN A CHILD WITH CHRONIC MUCOCUTANEOUS CANDIDIASIS AND AUTOSOMAL DOMINANT SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 1 GAIN OF FUNCTION (STAT1 GOF) MUTATION

D Chan,[1] J King,[1] L Dzeladini,[1] B Marchand,[1] M Gold[1,2] and P Quinn[1,2]

[1]Women's and Children's Hospital, Adelaide, Australia
[2]University of Adelaide, Adelaide, Australia

Background: Ruxolitinib is an oral tablet and is an inhibitor for Janus Associated Kinases (JAKs) JAK1 and JAK2. It is approved in Australia for the treatment of myelofibrosis. STAT1 GOF mutation was reported as a cause of autosomal dominant chronic mucocutaneous candidiasis (CMC) in 2011. Recent case reports with ruxolitinib have reported clinical benefit in alopecia areata and STAT1 GOF chronic mucocutaneous candidiasis.

Case report: We report our clinical experience in a 4-year-old child who is part of a large kindred affected by autosomal dominant CMC. She presented with onychomycosis, mild oral candida, and a large chronic ulcer of scalp. Scalp fungal scrapings grew aspergillus ornatus a soil fungus, and Lictheimia corymbifera. Fungal hyphae were seen on direct microscopy. Staphlococcus aureas and mixed growth including pseudomonas was also found.

Although we suspected autosomal dominant STAT1 GOF in distant family members they did not have a genetic diagnosis established. We performed Sanger sequencing on the index patient and detected a pathologic variant in STAT1 gene consistent with the diagnosis of STAT1 GOF.

Scalp debridement, standard antimicrobial therapy and antifungal therapy was implemented to treat the scalp ulcer. Given the severity of the disease, and lack of response, Ruxolitinib 5 mg nocte was added in as an adjuvant. Dosage was increased to 10 mg after one month. After 6 weeks of therapy significant improvement has been noted with hair regrowth, loss of hyperkeratotic crusting, and early skin regeneration. No significant side effects were noted.

Use of ruxolitinib or future JAK inhibitors may have a role in management of STAT1 GOF.

P79
INFECTED DERMATITIS ASSOCIATED WITH HTLV-1: A COPYCAT FOR CHILDHOOD CUTANEOUS CONDITIONS

B Marchand, D Rudaks, M Gold, D Chan and P Quinn

Womens and Childrens Hospital, Adelaide, Australia

Background: Infective dermatitis associated with HTLV-1 can easily be misdiagnosed as atopic dermatitis, putting patients at risk. Human T-Lymphotropic Virus type 1 (HTLV-1) is a single-stranded RNA retrovirus that preferentially infects CD4+ T cells. It is highly prevalent in the Australian Indigenous population. Transmission follows exposure to infected lymphocytes, which can occur via vertical and horizontal transmission, and importantly long-term breastfeeding. Infective dermatitis may not simply signal HTLV-1 infection, but is an indicator of increased risk of more devastating HTLV-1 disease complications. Known complications are rapidly progressive haematological malignancy (eg adult T-cell leukaemia/lymphoma), inflammatory disorders (e.g. myelopathy/tropical spastic paraparesis), pulmonary disease (eg bronchiectasis), as well as life threatening invasive *Staphylococcal* infections.

Case report: Our index case is a 2-year-old Indigenous female patient, referred with recurrent 'severe infected eczema,' requiring multiple admissions. Consideration had been given to STAT3 mutation, however, extensive workup, including immunological workup, lead to the diagnosis of HTLV-1 on blood PCR. Furthermore, she fulfilled the major criteria for the diagnosis of infective dermatitis associated with HTLV-1. Since diagnosis, when compliant with appropriate treatment, response has been dramatic.

Diagnosis: HTLV-1 infection is diagnosed on blood PCR. Prognostically, an HTLV-1c proviral load is essential.

In addition to a blood PCR, clinical criteria for diagnosis of infective dermatitis associated with HTLV-1 requires presence of skin lesions and chronic relapsing dermatitis recurring post cessation of systemic treatment, with or without crusting of nostrils.

Treatment: Treatment is aimed at controlling the complicating infections of *staphylococcus aureus* and *beta haemolytic streptococcus*. Long-term trimethoprim/sulfamethoxazole is recommended until at least adolescence.

Conclusion: Infective dermatitis associated with HTLV-1 is a clinically important mimic of other childhood dermatoses which requires high index of suspicion to ensure aggressive long term treatment to reduce morbidity and mortality.

P80
INTUSSCEPTION: A NOVEL PRESENTATION OF ACTIVATED PHOSPHOINOSITIDE 3-KINASE-Δ (PI3KΔ) SYNDROME (APDS)

M Gold,[1,2] D Chan,[1] B Marchand,[1] L Dzeladini,[1] J King,[1] A Cheung[1] and P Quinn[1]

[1]Women's and Children's Hospital, Adelaide, Australia
[2]University of Adelaide, Adelaide, Australia

Activated phosphoinositide 3-kinase-δ (PI3Kδ) syndrome (APDS) is recently described primary immunodeficiency disorder. The presentation is diverse and includes recurrent airway infections including bronchiectasis, ear and sinus infections, severe herpes infections, lymphadenopathy and a predisposition to B cell lymphoma.

The index patient (HC) is now 13 years of age. She presented at 4 years with an unexpected and life-threatening Adenoviral pneumonia requiring PICU and high frequency ventilation. Her elder sister (now 17 years) had a diagnosis of Common Variable Immunodeficiency Disorder with bronchiectasis and was receiving IVIG replacement (commenced at 5 years). Her mother (now aged 46 years) has a chronic history of recurrent upper respiratory tract infections.

Following this episode HC was demonstrated to have a Specific Antibody Deficiency and she was commenced on IVIG replacement. She remained reasonably well until 8 years of age when she presented with 6 weeks of weight loss and acute abdominal pain. Ultrasound demonstrated an

intussception with the ileum extending into the descending colon. Multiple well defined hypo-echoic lesions were demonstrated within the bowel wall suggesting a lymphoma. A laparotomy was performed and a very thickened distal terminal ileum and caecum was noted. No bowel biopsies were obtained because of concern about perforation. Biopsies from multiple lymph nodes showed reactive changes without clonality. The intussusception was reduced and she made an uneventful recovery. Subsequent morbidity has included marked sub-mental infective adenitis requiring surgical drainage on two occasions. She remains on monthly IVIG replacement without abdominal symptoms or signs. Annual ultrasound abdominal screening continues to demonstrate multiple mesenteric and bowel wall nodes.

Next generation sequencing in the index patient (HC), her sibling and mother show a heterozygous pathogenic variant (based on ACMG guidelines) in the PIK3CD gene (c.3061G>A : p.Glu1021Lys) which has been confirmed by Sanger sequencing. APDS is caused by heterozygous gain-of-function mutations in *PIK3CD* that induce hyper-activation of the protein products p110δ or p85α (respectively). APDS displays features of both immune deficiency and of immune dysregulation as demonstrated in this kindred.

P81
AN AUDIT OF SUBCUTANEOUS IMMUNOGLOBULIN REPLACEMENT THERAPY IN SYDNEY: 3 YEARS EXPERIENCE IN A SINGLE CENTRE

C Wijaya,[1] M Moussa,[2] M Rafferty[1] and S Limaye[1]

[1]Concord Hospital, Sydney, Australia
[2]Royal Prince Alfred Hospital, Sydney, Australia

Background: Immunoglobulin replacement therapy (IRT) is the standard of care for patients with humoral immunodeficiency and can be administered via the intravenous or subcutaneous route. Subcutaneous immunoglobulin (SCIg) is efficacious, well tolerated and results in more stable serum IgG levels compared with the peaks and troughs associated with intravenous immunoglobulin (IVIg). Home-based SCIg offers the advantage of improved autonomy and increased convenience for patients compared with intravenous institution-based therapy.

Methods: An audit of patient outcomes was conducted 3 years following the introduction of SCIg at Concord Hospital, Sydney.

Results: 11 patients were commenced on SCIg over 3 years, and 1 patient transferred from a paediatric centre. Of the 11 patients who initiated treatment at Concord, 1 withdrew prior to completion of training due to external factors. Patients were assessed as competent to self-administer SCIg after 3–6 weekly training sessions. SCIg products prescribed included Evogam (4 patients) and Hizentra (7 patients), using an approximate 1:1 dose conversion from IVIg product. Patients were provided with pumps and consumables. 3 patients required withdrawal of SCIg and recommencement of IVIg due to cutaneous reactions, life-threatening sepsis or patient preference. Trough levels of patients on SCIg will be presented.

At 3 years, 8 patients remain on SCIg, with half infusing once per week and half infusing on 2 days/week. Patients predominantly prefer administration via an infusion pump, with a minority of patients adopting the 'push' technique.

Conclusion: Although SCIg is a safe and established therapy, our audit reveals a relatively high rate of treatment withdrawal in a small group of patients with primary immunodeficiency. SCIg represents an effective method of IRT in carefully selected patients, although close monitoring of trough levels and adverse events is mandated.

P82
A NATIONAL AUDIT OF HEREDITARY AND AQUIRED ANGIOEDEMA IN NEW ZEALAND

K Lindsay,[1] P Flanagan,[2] P Fitzharris[1] and A Jordan[1]

[1]Auckland City Hospital, Auckland, New Zealand
[2]New Zealand Blood Service, Auckland, New Zealand

Hereditary Angioedema (HAE) is a rare potentially life threatening genetic condition, but access to effective therapies can reduce mortality and improve quality of life. Patients with this condition in New Zealand remain uncharacterised by number, geographical distribution, severity or treatment experience.

New Zealand Immunologists were invited to recruit patients with HAE and AAE to the audit or those identified as having Berinert® for hereditary or acquired angioedema. Participants were consented, their angioedema related health information was collected and they were invited to take part in telephone or face to face interviews about their experience of healthcare.

Twenty-one patients with hereditary angioedema were recruited, three of whom had acquired angioedema C1 inhibitor deficiency. Three patients were diagnosed prior to the onset of symptoms due to the diagnosis of a family member with the disease by screening family members. The average diagnostic delay was 12.9 years. Variation in delay between different types of HAE, with the greatest mean delay being in Type 1 HAE at 18.5 years, 13 years in type 2 HAE, and 3.6 years in AAE.

Within the cohort of 21 patients there were reports of 4 deaths of family members due to HAE. The majority of patients 19/21 (90%) had a written plan to present to the emergency department. Few (24%) had a Medic Alert® bracelet. In 2015 there were a total of 217 HAE attacks in 16 patients. Five patients (24%) were asymptomatic. Only one patient had angioedema of the upper airway in 2015 but did not require intubation. Six patients had 136 abdominal attacks; some with high frequency (range 1–52). Four patients said HAE had no impact on their life, 10 had minor impact, and 4 moderate and 3 described it as severe. This study characterises a cohort of AAE and HAE patients in New Zealand.

P83
QUALITY OF LIFE (QOL) ASSESSMENTS FOLLOWING THE USE OF PROPHYLACTIC C1 ESTERASE INHIBITOR CONCENTRATE IN PATIENTS WITH SEVERE HEREDITARY ANGIOEDEMA

A Merry, D Heyworth-Smith, C Kennedy, S Robertson and L James

Greenslopes Private Hospital, Brisbane, Australia

Introduction: Hereditary Angioedema (HAE) is a rare genetic condition resulting in a deficiency in C1 inhibitor protein and is characterised by both provoked and unprovoked episodes of angioedema. In a severe phenotype patients may experience repeated and frequent facial or limb angioedema, severe and recurrent abdominal pain and a risk of death by asphyxiation as a result of upper airway angioedema. The relentless nature of this condition with frequent and debilitating attacks along with previous 'on-demand' treatment requiring a hospital presentation for each attack has a profound impact on the quality of life for this patient group.

In 2016 the National Blood Authority (NBA) allowed for the routine supply of C1 inhibitor concentrate to HAE patients experiencing eight or more acute attacks per month. With appropriate training patients are able to self administer treatment at home thus avoiding hospital presentations. This change in treatment is predicted to provide a dramatic change to the QOL for this patient group.

Methods: HAE patients completed a HAE-QoL score pre and post the commencement of prophylactic use of C1 inhibitor concentrate, along with school/work attendance and documentation of disease activity.

Summary of results: Following prophylactic use of C1 inhibitor concentrate, we have seen a marked reduction in disease activity and resultant improvement in quality of the life as measured by HAE-QoL score and school/work attendance.

Conclusions: The availability of prophylactic use of C1 inhibitor concentrate in HAE patients with a severe phenotype has had a significant beneficial effect on the quality of life of this patient group.

P84
COMBINED IMMUNODEFICIENCY DUE TO DEFICIENCY OF ACTIN-RELATED PROTEIN COMPLEX 1B (ARPC1B)

P Quinn,[1,2] C Pearson,[1] A Cheung,[1] J King,[1,3] D Chan,[1] B Marchand,[1] D Rudaks,[1] A Quach,[3] A Ferrante[3] and M Gold[1,2]

[1]Women's and Children's Hospital, Adelaide, Australia
[2]University of Adelaide, Adelaide, Australia
[3]SA Pathology, Adelaide, Australia

A number of cytoskeletal defects arising from perturbations in actin polymerisation and resulting in combined primary immunodeficiency (PID) have been described. Defects include Wiskott Aldrich Syndrome (WAS), WAS Interacting Protein (WIP) and Dedicator of Cytokinesis 8 (DOCK-8) deficiencies. A newly reported cytoskeletal defect is Actin-related Protein Complex 1B (ARPC1B) deficiency. Here we report the first

Australian, youngest yet diagnosed, and 5th only reported case of ARPC1B deficiency.

A female infant born to Nepalese parents presented with bloody diarrhoea and metabolic acidosis at 2 months of age. A diagnosis of cow's milk enteritis was made and she was commenced on an elemental formula with resolution of the hematochezia and improvement in the diarrhoea. Subsequently she developed an eczematous rash which progressed to an erosive purpuric dermatitis with ulceration, and later took on a psoriasiform appearance. Skin biopsy showed a leucocytoclastic vasculitis. Her progress was complicated by cytomegalovirus infection, recurrent suppurative otitis media, twice by periorbital cellulitis, persistent oral candidiasis, urinary tract infection and failure to thrive.

Investigations showed defective neutrophil chemotaxis and random migration, reduced Natural Killer cell cytotoxicity, decreased naïve T-cells, variable thrombocytopenia, eosinophilia, and raised IgA and IgE. Whole exome sequencing showed her to be homozygote for an exon 2 splice site mutation in ARPC1B (c.64+2T>A) which is predicted to cause loss of gene function. Western blot analysis showed low expression of the ARPC1B protein.

ARPC1B is a regulatory subunit of the actin-related protein 2/3 complex critical for F-actin polymerisation in haemopoetic cells. Deficiency of ARPC1B results in defective actin polymerisation and a combined immunodeficiency similar but distinct to WAS.

P85

SELF-INFUSION OF BERINERT® (HUMAN C1-ESTERASE INHIBITOR) IN THE HOME. EARLY LEANINGS FROM BERINERT NURSECARE, A HOME BASED EDUCATION PROGRAM TO TEACH PATIENTS HOW TO SELF-INFUSE

D Tognarini, E Vagg and S Youssef

Aesir Health, Cheltenham, Victoria, Australia

Hereditary Angioedema (HAE) is a rare condition, affecting approx. 1 in 50 000, and knowledge within the general medical community (i.e. outside Immunology clinics) is low. To support patients & clinics, the ASCIA HAE Positioning Paper (2017) recommends that each HAE sufferer has a HAE Action Plan highlighting when to initiate home based self-administrated medication and when to escalate to ED.

In October 2016, the HAE treatment paradigm changed significantly in Australia with the NBA funding of C1- INH concentrate. However, the question remained regarding feasibility regarding home based preparation and infusion.

Aesir Health (independent provider with 13+ years' experience educating patients on self-administered sub-cutaneous injectables) commissioned by CSL Behring, designed, built and is managing the Berinert NurseCare Educational Program (initiated in Feb 2017). This national program has objectives to ensure concordance with BERINERT® (Human C1-Esterase Inhibitor) prescribing on several levels including:

- competence in reconstitution
- accurate vein identification, site rotation & aseptic venepuncture
- correct administration, post procedure care and documentation
- clarity of patient's HAE Action Plan including customisation with home circumstances and support networks

Currently, 16 patients are enrolled, requiring an average of 2.5 visits per patient and 4.8 total hrs per education per patient. Some attributes facilitating uptake and acceptance to home-infusion include having direct family/carer support networks, experiencing recent HAE attacks, poor experience at ED. Learning is complimented by using novel training apparatus plus the opportunity to prepare & administer BERINERT.

These initial findings suggest that home based infusion is feasible and further investigation into the elements which patients find challenging or supportive is warranted.

P86

DEVELOPING A HOME-BASED MODEL OF CARE FOR ADMINISTRATION OF SUBCUTANEOUS IMMUNOGLOBULINS

J Waymouth, A-M Redden and J Pollard

University Hospital Geelong, Geelong, Australia

Aims: To develop a model of care within the Barwon Health (BH) Hospital in the Home (HITH) program to transition patients from a day stay infusion model of intravenous immunoglobulin (IVIg) to a self-administered home based model of care - subcutaneous immunoglobulin (SCIg). The program is available to all eligible patients currently receiving IVIg in BH day infusion centres and who reside within the local catchment of BH and the surrounding regional communities.

Methods: A model of care has been developed based on previous individual patient approaches to teaching self-management of IVIg infusions with ongoing HITH support and facilitation. This program has required further development and scaling up of existing processes to support a model of care that can support multiple patients in a systematic, efficient and sustainable manner.

This model has adopted a collaborative approach with the support of CSL product advisors and educators, treating clinicians, the BH transfusion services clinical nurse specialists, existing BH day infusion and pharmacy services.

Patient education modules, education brochures, SCIg specific care plans and processes have been developed to support the model of care.

The transition from IVIg to SCIg is occurring with initial treatment and education in the HITH clinic, followed by treatment and education in the home setting, Dependent on patient competency and confidence it is envisaged that most patients will transition to self-management within 4–6 weeks. HITH will provide ongoing support and education with planned reviews and facilitation of product ordering and supply and provision of consumables.

Results: The program is currently in the early stages of implementation. A questionnaire is being used to measure patient experience and satisfaction. An evaluation of the program based on number of patient enrolments, reduction in health service costs and treating clinician engagement and satisfaction of treatment efficacy will be utilised to support this approach to care.

Conclusion: The HITH approach to the implementation of a SCIg program is an important initiative providing patients with an alternative and self-management care option.

COI Disclosure: JW-Financial support to attend the event was provided by CSL Behring

P87

USE OF RITUXIMAB TO TREAT MOOREN'S ULCER

N Benwell and A McLean-Tooke

Sir Charles Gairdner Hospital, Perth, Australia

Introduction: Peripheral ulcerative keratitis (PUK) can be a secondary manifestation of systemic autoimmune diseases, most commonly Rheumatoid arthritis (RA) and Granulomatosis Polyangiitis GPA). PUK can precede systemic symptoms and its presence may herald sight threatening if not life threatening disease. Mooren's ulcer is a rare form of idiopathic PUK, whose aetiology includes autoimmunity and loss of tolerance to components within the corneal stroma. Whilst biologic agents have been successful in treating systemic disease and PUK in RA and GPA, their role in treating PUK in the absence of systemic autoimmune diseases remains less clear.

Case report: We present the case of a 16-year-old female who developed sudden onset bilateral painful ulcerative keratitis. She was diagnosed with Mooren's ulcer in absence of evidence of systemic autoimmune disease on clinical examination or autoimmune serology.

Despite topical and systemic steroid therapy, she developed 'corneal melt' requiring an urgent tectonic corneal graft and with IV methylprednisolone, oral prednisolone and mycophenolate mofetil to stabilise disease. Her disease course was characterised by fluctuating visual acuity and progressive corneal thinning with recurrent disease within the corneal graft and progression toward re-perforation despite ongoing immunosuppression. She was given a trial of rituximab given the aggressive and refractory nature of

The Role of Phagocytes in Immunity to *Candida albicans*

Annabelle Small [1,2]

Jovanka R. King [1,3]

Deborah Rathjen [1,4]

*Antonio Ferrante [1,2]

[1] Department of Immunopathology, SA Pathology at Women's and Children's Hospital Campus, The Robinson Research Institute and School of Medicine, University of Adelaide, South Australia, 5000.

[2] School of Biological Science, University of Adelaide, South Australia, 5000.

[3] Division of Clinical Immunology, Department of Laboratory Medicine, Karolinska University Hospital Huddinge, SE-14186 Stockholm, Sweden.

[4] DAR, Bionomics, Pty Ltd, Thebarton, South Australia, 5031.

* Corresponding Author

Abstract

Clearance of fungal pathogens such as Candida albicans from the human body involves their phagocytosis by fixed tissue macrophages as well as infiltrating monocytes and neutrophils. Through an efficient phagocytotic process the fungi are confined and killed by the oxidative and non-oxidative anti-microbial components of the cells. These include oxygen derived reactive species, generated from the activation of the NADPH oxidase complex, together with the release of myeloperoxidase to generate hypochlorous acid. Assisting phagocytosis is the complement system which opsonises fungi to promote their interaction with a series of complement receptors including the recently described complement receptor immunoglobulin. Phagocytosis and killing of Candida can be enhanced by the cytokine network of the immune system. Cytokines such as tumour necrosis factor and interferon gamma prime phagocytes and increase their anti-microbial power. Patients with primary immunodeficiency diseases who have phagocytic defects, such as those with defects in the NADPH oxidase complex are predisposed to fungal infections, providing evidence for the critical role of phagocytes in anti-fungal immunity. Secondary immunodeficiencies can arise as a result of treatment with anti-cancer or other immunosuppressive drugs. These may also predispose patients to fungal infections due to their ability to compromise the anti-microbial activity of phagocytes.

REFERENCES

1. Bousfiha A, Jeddane L, Picard C, et al. The 2017 IUIS Phenotypic Classification for Primary Immunodeficiencies. *J Clin Immunol* 2018; 38(1): 129-43.

2. Gaspar HB, Hammarström L, Mahlaoui N, Borte M, Borte S. The case for mandatory newborn screening for severe combined immunodeficiency (SCID). *J Clin Immunol* 2014; 34(4): 393-7.

3. Adeli MM, Buckley RH. Why newborn screening for severe combined immunodeficiency is essential: a case report. *Pediatrics* 2010; 126(2): e465-9.

4. Buckley RH. The long quest for neonatal screening for severe combined immunodeficiency. *J Allergy Clin Immunol* 2012; 129(3): 597-604; quiz 5-6.

5. Dvorak CC, Cowan MJ, Logan BR, et al. The natural history of children with severe combined immunodeficiency: baseline features of the first fifty patients of the primary immune deficiency treatment consortium prospective study 6901. *J Clin Immunol* 2013; 33(7): 1156-64.

6. Pai SY, Logan BR, Griffith LM, et al. Transplantation outcomes for severe combined immunodeficiency, 2000-2009. *N Engl J Med* 2014; 371(5): 434-46.

7. Kwan A, Abraham RS, Currier R, et al. Newborn screening for severe combined immunodeficiency in 11 screening programs in the United States. *JAMA* 2014; 312(7): 729-38.

8. Wilson JM, Jungner YG. Principles and practice of mass screening for disease. *Bol Oficina Sanit Panam* 1968; 65.

9. Chan K, Puck JM. Development of population-based newborn screening for severe combined immunodeficiency. *J Allergy Clin Immunol* 2005; 115(2): 391-8.

10. Borte S, von Döbeln U, Fasth A, et al. Neonatal screening for severe primary immunodeficiency diseases using high-throughput triplex real-time PCR. *Blood* 2012; 119(11): 2552-5.

11. van Zelm MC, Szczepanski T, van der Burg M, van Dongen JJ. Replication history of B lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. *J Exp Med* 2007; 204.

12. Mensen A, Ochs C, Stroux A, et al. Utilization of TREC and KREC quantification for the monitoring of early T- and B-cell neogenesis in adult patients after allogeneic hematopoietic stem cell transplantation. *J Transl Med* 2013; 11: 188.

13. Kamae C, Nakagawa N, Sato H, et al. Common variable immunodeficiency classification by quantifying T-cell receptor and immunoglobulin kappa-deleting recombination excision circles. *J Allergy Clin Immunol* 2013; 131(5): 1437-40 e5.

14. Atschekzei F, Ahmad F, Witte T, Jacobs R, Schmidt RE. Limitation of Simultaneous Analysis of T-Cell Receptor and kappa-Deleting Recombination Excision Circles Based on Multiplex Real-Time Polymerase Chain Reaction in Common Variable Immunodeficiency Patients. *International archives of allergy and immunology* 2016; 171(2): 136-40.

15. Serana F, Airò P, Chiarini M, et al. Thymic and bone marrow output in patients with common variable immunodeficiency. *J Clin Immunol* 2011; 31.

16. Edgar JD, Buckland M, Guzman D, et al. The United Kingdom Primary Immune Deficiency (UKPID) Registry: report of the first 4 years' activity 2008-2012. *Clin Exp Immunol* 2014; 175(1): 68-78.

17. Plebani A, Soresina A, Rondelli R, et al. Clinical, immunological, and molecular analysis in a large cohort of patients with X-linked agammaglobulinemia: an Italian multicenter study. *Clin Immunol* 2002; 104(3): 221-30.

18. Winkelstein JA, Marino MC, Lederman HM, et al. X-linked agammaglobulinemia: report on a United States registry of 201 patients. *Medicine* 2006; 85(4): 193-202.

19. Janzi M, Sjöberg R, Wan J, et al. Screening for C3 deficiency in newborns using microarrays. *PLoS One* 2009; 4(4): e5321.

20. Borte S, Janzi M, Pan-Hammarstrom Q, et al. Placental transfer of maternally-derived IgA precludes the use of guthrie card eluates as a screening tool for primary immunodeficiency diseases. *PloS one* 2012; 7(8): e43419.

21. Borte S, Meeths M, Liebscher I, et al. Combined newborn screening for familial hemophagocytic lymphohistiocytosis and severe T- and B-cell immunodeficiencies. *J Allergy Clin Immunol* 2014; 134(1): 226-8.

22. Jennewein MF, Abu-Raya B, Jiang Y, Alter G, Marchant A. Transfer of maternal immunity and programming of the newborn immune system. *Seminars in Immunopathology* 2017; 39(6): 605-13.

23. Willig LK, Petrikin JE, Smith LD, et al. Whole-genome sequencing for identification of Mendelian disorders in critically ill infants: a retrospective analysis of diagnostic and clinical findings. *Lancet Respir Med* 2015; 3(5): 377-87.

24. Kingsmore SF. Newborn testing and screening by whole-genome sequencing. *Genet Med* 2016; 18(3): 214-6.

25. Smith LD, Willig LK, Kingsmore SF. Whole-Exome Sequencing and Whole-Genome Sequencing in Critically Ill Neonates Suspected to Have Single-Gene Disorders. *Cold Spring Harb Perspect Med* 2015; 6(2): a023168.

26. Petrikin JE, Willig LK, Smith LD, Kingsmore SF. Rapid whole genome sequencing and precision neonatology. *Semin Perinatol* 2015; 39(8): 623-31.

27. Al-Mousa H, Al-Dakheel G, Jabr A, et al. High Incidence of Severe Combined Immunodeficiency Disease in Saudi Arabia Detected Through Combined T Cell Receptor Excision Circle and Next Generation Sequencing of Newborn Dried Blood Spots. *Front Immunol* 2018; 9: 782.

28. Pavey AR, Bodian DL, Vilboux T, et al. Utilization of genomic sequencing for population screening of immunodeficiencies in the newborn. *Genet Med* 2017.

29. Hu L, Crawford SE, Czako R, et al. Cell attachment protein VP8* of a human rotavirus specifically interacts with A-type histo-blood group antigen. *Nature* 2012; 485(7397): 256-9.

30. Kambhampati A, Payne DC, Costantini V, Lopman BA. Host Genetic Susceptibility to Enteric Viruses: A Systematic Review and Metaanalysis. *Clin Infect Dis* 2016; 62(1): 11-8.

31. Nordgren J, Sharma S, Bucardo F, et al. Both Lewis and secretor status mediate susceptibility to rotavirus infections in a rotavirus genotype-dependent manner. *Clin Infect Dis* 2014; 59(11): 1567-73.

32. King J, Ludvigsson J, Hammarström L. Newborn Screening for Primary Immunodeficiency Diseases: The Past, the Present and the Future. *International Journal of Neonatal Screening* 2017; 3(3): 19.

33. King JR, Hammarström L. Newborn Screening for Primary Immunodeficiency Diseases: History, Current and Future Practice. *Journal of Clinical Immunology* 2017.

34. Khoo SK, Dykema K, Vadlapatla NM, et al. Acquiring genome-wide gene expression profiles in Guthrie card blood spots using microarrays. *Pathology international* 2011; 61(1): 1-6.

35. Gauffin F, Nordgren A, Barbany G, Gustafsson B, Karlsson H. Quantitation of RNA decay in dried blood spots during 20 years of storage. *Clinical chemistry and laboratory medicine* 2009; 47(12): 1467-9.

36. Karlsson H, Guthenberg C, von Dobeln U, Kristenssson K. Extraction of RNA from dried blood on filter papers after long-term storage. *Clin Chem* 2003; 49(6 Pt 1): 979-81.

37. Kaneko H, Kondo N, Motoyoshi F, et al. Expression of immunoglobulin genes in common variable immunodeficiency. *Journal of Clinical Immunology* 1991; 11(5): 262-7.

38. Barbany G, Gauffin F, Ofverholm I, et al. The ETV6/RUNX1 fusion transcript is not detected in RNA isolated from neonatal dried blood spots from children later diagnosed with the corresponding leukemia. *Leuk Lymphoma* 2013; 54(12): 2742-4.

39. Wilson J, Jungner G. The principles and practice of screening for disease. Geneva: World Health Organization; 1968.

40. Villoria JG, Pajares S, López RM, Marin JL, Ribes A. Neonatal Screening for Inherited Metabolic Diseases in 2016. *Seminars in Pediatric Neurology* 2016; 23(4): 257-72.

41. Clague A, Thomas A. Neonatal biochemical screening for disease. *Clinica Chimica Acta* 2002; 315(1): 99-110.

42. Therrell BL, Padilla CD, Loeber JG, et al. Current status of newborn screening worldwide: 2015. *Seminars in Perinatology* 2015; 39(3): 171-87.

43. Grazioli S, Bennett M, Hildebrand KJ, Vallance H, Turvey SE, Junker AK. Limitation of TREC-based newborn screening for ZAP70 Severe Combined Immunodeficiency. *Clin Immunol* 2014; 153(1): 209-10.

44. Kuo CY, Chase J, Garcia Lloret M, et al. Newborn screening for severe combined immunodeficiency does not identify bare lymphocyte syndrome. *J Allergy Clin Immunol* 2013; 131(6): 1693-5.

45. Speckmann C, Neumann C, Borte S, et al. Delayed-onset adenosine deaminase deficiency: strategies for an early diagnosis. *J Allergy Clin Immunol* 2012; 130(4): 991-4.

46. van Zelm MC, Berkowska MA, van Dongen JJ. Studying the replication history of human B lymphocytes by real-time quantitative (RQ)-PCR. *Methods Mol Biol* 2013; 971: 113-22.

47. Nakagawa N, Imai K, Kanegane H, et al. Quantification of κ-deleting recombination excision circles in Guthrie cards for the identification of early B-cell maturation defects. *J Allergy Clin Immunol* 2011; 128.

48. Currier RL, Payne DC, Staat MA, et al. Innate Susceptibility to Norovirus Infections Influenced by FUT2 Genotype in a United States Pediatric Population. *Clin Infect Dis* 2015; 60(11): 1631-8.

49. Payne DC, Currier RL, Staat MA, et al. Epidemiologic Association Between FUT2 Secretor Status and Severe Rotavirus Gastroenteritis in Children in the United States. *JAMA Pediatr* 2015; 169(11): 1040-5.

50. Friedman JM, Cornel MC, Goldenberg AJ, et al. Genomic newborn screening: public health policy considerations and recommendations. *BMC Med Genomics* 2017; 10(1): 9.